THE METABOLISM OF INTRAVENOUS TESTOSTERONE

Charles D. West

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Department of Biochemistry

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Leo T. Samuels
Department of Biochemistry

Henry Eyring
Dean, Graduate School

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INTRODUCTION

Although it has never been isolated from blood, testosterone is usually considered to be the circulating androgen secreted by the testis for the following reasons: (a) Castration effects are relieved by testosterone administration. (b) Testosterone is the only true androgen that has been isolated from testis tissue. (c) It is the most potent of all known androgens.

In recent years investigation of the metabolism of testosterone has assumed great importance, not only because of its androgenic effects, but also because of its marked influence on the growth of tissue, both normal and neoplastic.

One method of study has involved the isolation of metabolites from the excretions following the administration of testosterone to animal or human subjects. Because of its low solubility in aqueous solution, testosterone could not be administered intravenously and had to be given orally, subcutaneously, or intramuscularly. These routes of administration had the disadvantages of slow absorption and utilization. It was difficult to define accurately the metabolites of testosterone because of interfering endogenous steroids that were excreted during the experimental period. Only a small percentage of the administered testosterone could be accounted for.

With the recent demonstration by Bischoff (1) of the high solubility of testosterone in serum albumin, it became possible to administer large doses of testosterone dissolved in serum albumin

intravenously and to study for the first time the fate, distribution, and excretion of circulating testosterone. It was also postulated that a study of the metabolism of intravenous testosterone should yield valuable information because the disadvantages of slow absorption and utilization were removed.

For these reasons an investigation of the metabolism of testosterone administered intravenously to intact animals and human subjects was undertaken.

HISTORICAL REVIEW

The development of the concept of the testis as an endocrine organ can be traced to ancient times. Castration on both animals and man was then a common practice and effects on the secondary sex characteristics were noted.

The first experimental demonstration of the hormonal action of the testes was performed by John Hunter (2) in 1794 in which he showed that the rudimentary spur of a hen, when transplanted to the leg of a cock, would grow into a mature masculine spur. Furthermore, Hunter believed that the accessory genital organs depended upon the testes for their maintainance.

These observations were overlooked until 1849 when Berthold (3) demonstrated that the castration effects in capons could be relieved by testicular transplants.

Brown-Sequard (4) (1889) at the age of 72 injected himself with saline extracts prepared from dog and guinea pig testes and claimed "increased bodily and mental vigour and an improvement of intestinal tone."

Although these effects were probably mainly from autosuggestion, sufficient interest was aroused by these experiments to start other investigators working in the field.

Loewy (5) (1903) was perhaps the first to observe objective changes in castrated animals following the administration of testicular extracts. When injected into young capons, these extracts caused skeletal and comb development typical of the male.

Pezard (6) (1911) observed the same masculinizing effects upon capons by the injection of extracts of cryptorchid testes from pigs.

A new era in the isolation studies of the testis hormone began in 1927 when McGee (7) prepared from bulls' testes a relatively pure lipid extract having androgenic potency. A year later Loewe and Voss (8) (1928) demonstrated the presence of androgen in urine.

This new era was made possible by the development of methods of analysis for androgens which enabled investigators to follow the degree of purification of their extracts when subjected to various procedures. McGee first utilized the growth of the capon's comb as a qualitative and semi-quantitative test for androgens while preparing his potent lipid extracts of testes. In 1930 Gallagher and Koch (9) established comb growth as a quantitative reproducible assay for androgens.

Other less convenient and more time consuming methods depending upon the growth and histological changes in the prostate and seminal vesicle of the castrated rat were also developed (10-11).

Zimmermann(12-13) in 1935 developed a convenient chemical method for quantitatively measuring ketonic steroids in body fluids. This was a colorimetric method, depending upon the development of a characteristic color with alkaline m-dinitrobenzene. It has undergone several modifications since first described. The most commonly used today are the Callow (14) and Holtorff and Koch (15) modifications. Callow found a good correlation between this method and the androgenic assay.

With these methods of analysis many different laboratories were able to prepare highly purified extracts, both from urine and testis tissue. In 1931 Butenandt (16-17) announced the first isolation of

crystalline material having androgenic potency from concentrates of men's urine. Three different crystalline substances were obtained, and later proved to be androsterone, dehydroisoandrosterone, and the chloro derivative of dehydroisoandrosterone. The structures of androsterone and dehydroisoandrosterone were established by partial synthesis from cholesterol (14-19-20).

It became apparent that still a third and different androgen must be present in testis tissue. Gallagher and Koch (21) had shown that the testis extract androgen was more sensitive to alkali than the urinary androgens. Dingemanse et al. (22) had shown that highly purified extracts of bull's testes were more active on a weight basis than the pure compounds isolated from urine.

Finally in 1935 David, Dingemanse, Freud, and Laqueur (23) isolated testosterone in a crystalline form from bulls' testes. Testosterone had six times the androgenic activity of androsterone as well as different chemical properties. David's isolation was confirmed by Ruzicka (24) who isolated testosterone from the testes of stallions.

The synthesis of testosterone from cholesterol and proof of structure was accomplished by Ruzicka and Wettstein (25) and by Butenandt and Kudszus (26).

Other crystalline compounds have been isolated from testis tissue. Ruzicka and Prelog (27) have demonstrated two C21 steroids in boar testis tissue, $\overset{5}{\Delta}$pregnenol-3β-one-20 and allopregnanol-3β-one-20. Two isomeric androstenols, $\overset{16}{\Delta}$-androstenol-3α and $\overset{16}{\Delta}$-androstenol-3β were also isolated from swine testes.

Testalone, an inactive substance having an empirical formula

$C_{21}H_{32}O_3$, was first isolated from swine testes by Hirano (29) and confirmed by Ruzicka and Prelog (30). Its structure is unknown.

Hirano isolated a second substance from testis tissue with the formula $C_{19}H_{40}O_3$. Prelog, Ruzicka, and Steinman (31) later isolated the same substance from swine testes and proved it to be chimyl alcohol.

Testosterone is the only androgenic substance so far isolated from testis tissue.

That the testicular androgen was elaborated by the interstitial cells of the testis was shown early. Bouin and Ancel (32-33) as early as 1903 had shown that vasoligation causes the seminal epithelium to atrophy but leaves the Leydig cells intact. Under these conditions the accessory genital organs maintained their normal size. Moore (34) demonstrated that a cryptorchid testis secretes as much androgen as a normal testis although it weighs only 2.8% as much and contains no seminiferous tubules. Witschi (35) et al demonstrated that x-radiation of rat testes destroyed the seminal epithelium and left the interstitial cells intact. The prostates and seminal vesicles in these animals were normal. Finally it has been demonstrated that interstitial cell tumors in the human produce large amounts of androgen (36). Evidence therefore indicated that the source of androgens in the testis is the interstitial tissue.

In the course of the investigation of the androgens it became apparent that testicular tissue was not the only source of androgens. Callow and Callow (37) found that the total amount of

androgen excreted by a twenty year old eunuch was about the same as that excreted by a normal man. However, the androsterone and etiocholanol-3α-one-17 observed in the normal was largely replaced by dehydroisoandrosterone in the eunuch.

Androgens have been demonstrated also in urine from females as well as from males (38). Callow and Callow (39) were able to demonstrate the same androgens in normal female urine as in normal male urine in much the same distribution. Perhaps the best evidence that the ovary is capable of secreting androgens came from the experiments of Hill and Gardner (40). They grafted mouse ovaries into the ears of castrated male mice. As a result of the grafts the accessory genital organs instead of undergoing the usual post-castration atrophy were maintained in full functional activity (41-42). Parkes (43) has shown that lipid extracts of sow ovarian tissue possess androgenic activity. In spite of the convincing physiological demonstrations that the ovary can secrete an androgen, none has been isolated and identified from ovarian tissue.

Another possible source for the androgen in the urine of females, eunuchs, and castrated males is the adrenal gland. Lespinasse (44) in 1924 was the first to demonstrate that the adrenal was a source of androgens. He implanted mature adrenal tissue into the pectoral muscles of immature cockerels and thereby induced precocious male development and behavior.

The syndrome of masculinization in immature males and females associated with hypertrophy of the adrenal gland has long been recognized

by the clinicians (45-46).

In 1936 Reichstein (47-48) isolated and identified adrenosterone from adrenal tissue. It was a C19 steroid having about one-fifth the androgenic capacity of androsterone as measured by capon comb growth. Following this isolation other androgens, including androstanediol 3β, 11β,-one-17 (49), Δ^4androstenedione,3,17 (50), and 17-"β" hydroxyprogesterone (51-52) were isolated from adrenal cortical extracts by various laboratories. The possibility still exists, as recognized by Reichstein, that these C19 steroids are not present as such in the gland but are formed during the process of isolation.

Patients with adrenal hyperfunction and virilism usually show an increase in output of urinary androgens and 17-ketosteroids but not invariably (53). In 1936 Callow (54) reported the isolation of massive amounts of dehydroisoandrosterone from the urine of a six year old girl suffering from an adrenal cortical cancer.

In 1938 Butler and Marrian (55) isolated isoandrosterone from the urine of a patient with hypercorticism showing virilism. From the urine of a woman with an adrenal tumor Wolfe, Fieser, and Friedgood (56) isolated five different 17-ketosteroids, either in themselves androgenic, or closely related to known androgens. These 17-ketosteroids were androsterone, etiocholanol-3α-one-17, dehydroisoandrosterone, $\Delta^{3,5}$androstadienone-17, and androstanol-3α-one-17.

Mason and Kepler (57), from the urine of female cases of either adrenal tumor or adrenal hyperplasia, isolated the following androgens or related compounds: dehydroisoandrosterone, androsterone, etiocholanol-

3α-one 17, 11-hydroxyandrosterone, and Δ^5androstenediol-3β,17α.

Hirschmann (58) first isolated Δ^5androstenetriol 3β,16,17 from the urine of a seven year old boy with carcinoma of the adrenal cortex. This was later confirmed by Mason and Kepler (59). Although this compound is inactive androgenically it is of interest because of its relationship to dehydroisoandrosterone. A similar relationship has been observed between estrone and estriol.

From these isolations of large amounts of androgens and related compounds from cases of hypercorticism evidence accumulated that the adrenal cortex might elaborate androgen. Even more conclusive evidence followed when Callow (60) showed that women with bilateral oophorectomies were still able to excrete androgens into their urine. From the combined urines of nine women below the age of menopause who had undergone bilateral oophorectomies Hirschmann (61) was able to isolate two androgens, androsterone and dehydroisoandrosterone.

Animal experimental work has supported the concept that the adrenal cortex elaborates androgens.

In mice the secretion of androgens by the adrenal cortex has been attributed to an inner histological zone called the "x-zone" (62). Upon castration of the male mouse this zone hypertrophies (63). Testicular grafts (64) or the administration of various crystalline androgens (65) will prevent this hypertrophy.

Davidson and Moon (66) gave ACTH that contained no gonadotrophin to normal and castrated immature male rats. Their results showed not only an increase in the size of the adrenals but also a considerable increase in the accessory genital organs over those of untreated castrated

rats. There was no effect of the ACTH preparation on the accessory reproductive organs in adrenalectomized animals.

These physiological studies leave little doubt that the adrenal cortex is a source of androgens. From isolation studies it appears that the adrenal cortex, as well as testosterone, can give rise to dehydroisoandrosterone, androsterone, etiocholanol-3α-one17, and isoandrosterone in the urine. The role of the adrenal cortex must be considered in any study of testosterone metabolism in the whole animal.

The only other organ involved in the formation of androgens is the placenta. Extracts of human placental tissue have been shown to contain androgenic material by bioassay (67).

Besides those urinary androgens and related compounds previously considered, many others have been found in the urine.

Butenandt and Dannenbaum (68) first isolated etiocholanol-3α-one-17 in 1934 from the urine of normal men. Callow and Callow (69-70) also isolated the same substance from the urine of normal women and eunuchs.

Butler and Marrian (55) succeeded in isolating a third isomer of androsterone, isoandrosterone, from the urine of a patient with carcinoma of the adrenal cortex. Pearlman (71-72) isolated this isomer not only from urines of patients with adrenal hyperfunction but also from normal urine.

The fourth isomer, etiocholanol 3β-one17, was finally isolated in small amounts from pooled urines by Lieberman and Dobriner (73) et al. The latter investigators, in their exhaustive study of urinary steroids, isolated other compounds, not all androgenic, but closely related to known androgens and thus of interest in a consideration of the metabolism

of testosterone. These include androstanedione-3,17, etiocholanedione-3,17, Δ^4androstenedione-3,17, Δ^9androstenol-3α-one-17, Δ^9etiocholenol 3α-one-17, androstanediol-3α,11β-one-17 and etiocholanol-3α-dione 11,17 (74). In a later study they isolated in addition testosterone, etiocholanediol-3α,11β-one-17, androstanol-3α-dione 11,17, and etiocholanediol-3α,17β-one-11.

Etiocholanediol-3α,17α was first isolated from the urine of normal men by Butenandt et al (75). The 17 isomer has never been found. Neither has androstanediol-3α,17β been found in normal urine.

Δ^5androstenediol-3β,17α, a compound closely related to dehydroisoandrosterone , has been found in cases of adrenal tumor (76).

Urine is the only body excretion in which isolation and identification of steroids has been accomplished. Both bile (77-78) and feces (79-80) have been demonstrated to contain androgenic material but no isolations are reported.

Similarly, no isolations are reported from blood or somatic tissues although androgenic activity has been demonstrated in both (81-82-83).

Thus far we have considered all the androgens and closely allied substances which have been isolated from natural sources. Table I lists these compounds together with their source and androgenicity. All of these compounds are neutral steroids. With the exception of 17 "β"hydroxyprogesterone they all contain nineteen carbon atoms. All possess oxygen in positions C3 and C17. A few having oxygen on C11 are thought to arise from C11 oxygenated adrenal cortical steroids.

Some of these compounds are considered artifacts arising from

TABLE I

INDEX NO.	NAME OF COMPOUND	SOURCE	AMT. (γ) EQUAL TO 1 I.U.
I.	$^{4}_{A}$ androstenol-17α-one$_{3}$ (testosterone)	testis, normal urine	15
II	androstanol-3α-one-17 (androsterone)	normal urine	100
III	$^{5}_{A}$ androstenol-3β-one-17 (dehydroisoandrosterone)	normal urine adrenal ca. urine	700
IV	3-chloro- $^{5}_{A}$-androstenone-17	normal urine (artefact?)	inactive
V	$^{5}_{A}$ pregnenol-3β one-20	testis	inactive
VI	Allopregnanol-3β one-20	testis	inactive
VII	$^{16}_{A}$ androstenol-3α	testis	inactive
VIII	$^{16}_{A}$ androstenol-3β	testis	inactive
IX	etiocholanol-3α-one-17	normal urine	inactive
X	$^{4}_{A}$ androstenetrione-3,11,17 (adrenosterone)	adrenal cortex	500
XI	androstanediol-3β, 11β- one-17	adrenal cortex	"positive"
XII	$^{4}_{A}$ androstenedione-3,17	adrenal cortex & normal urine	100

TABLE I (CONTINUED)

INDEX NO.	NAME OF COMPOUND	SOURCE	AMT. (γ) EQUAL TO 1 I.U.
XIII	$^{4}_{\Delta}$ pregnenol-17β dione-3,20 (17-"β" hydroxyprogesterone)	adrenal cortex	500
XIV	androstanol-3β- one 17 (isoandrosterone)	normal urine adrenal ca urine	700
XV	$^{3,5}_{\Delta}$ androstadienone-17	normal urine (artefact?)	
XVI	$^{?}_{\Delta}$ androstenol 3α- one-17	normal urine	
XVII	androstanediol-3α,11-one-17 (11 hydroxyandrosterone)	urine of adrenal c.a.	300
XVIII	$^{5}_{\Delta}$ androstenediol-3β, 17α	urine of adrenal c.a.	
XIV	$^{5}_{\Delta}$ androstenetriol-3β,16,17	urine of adrenal c.a.	inactive
XX	etiocholanol -3β-one-17	normal urine	
XXI	androstanedione-3,17	normal urine	
XXII	etiocholanedione-3,17	normal urine	

TABLE I (CONTINUED)

INDEX NO.	NAME OF COMPOUND	SOURCE	AMT. (γ) EQUAL TO 1.I.U.
XXIV	$^{9}_{\Delta}$ androstenol - 3α- one 17	normal urine	
XXV	$^{9}_{\Delta}$ etiocholenol-3α one-17	normal urine (artefact?)	300
XXVI	etiocholanol - 3α, dione-11,17	normal urine	
XXVII	etiocholanediol-3α, 11β- one-17	normal urine	
XXVIII	androstanol-3α, dione - 11,17	normal urine	
XXIX	etiocholanediol - 3α, 17β- one-11	normal urine	
XXX	etiocholanediol - 3α, 17α	normal urine	
XXXII	$^{2}_{\Delta}$ or ^{3} androstenone-17	normal urine (artefact?)	1000

chemical manipulations during isolation, such as $\Delta^{2 \text{ or } 3}$androstenone-17 (36) and 3-chloro-Δ^{5}androstenone-17. C9 unsaturated steroids are thought to arise from 11-oxygenated steroids.

From a quantitative standpoint androsterone and etiocholanol-3-one-17 make up the bulk of the neutral urinary 17-ketosteroids (84). In most studies these two have occurred in approximately equal amounts and amount to from 70-90% of the neutral α-17-ketosteroids isolated (70-71-85). Dehydroisoandrosterone is the next most common 17-ketosteroid isolated, accounting for approximately another 10% of the 17-ketosteroids. All the other neutral C19 urinary steroids are present in normal urine in small amounts, requiring large collection pools for isolation.

The problem arose as to which of these urinary androgens and related compounds were metabolites of testosterone. One obvious method of studying this problem was to determine which components of the urine were increased after testosterone administration. Those present in amounts greater than normally expected could be considered as metabolites of testosterone.

The most active workers using this approach to the problem have been Callow, Dorfman, and indirectly, Mason.

Prior to their isolation studies many different investigators had demonstrated that the administration of testosterone increased the urinary androgens (86-87-88) as judged by bioassay.

Both Callow (89) and Dorfman (90) in 1939 reported the isolation of androsterone in increased amounts following the administration of testosterone propionate to hypogonadal males. In addition, having developed a chromatographic method of purification, Callow isolated increased

Amounts of etiocholanol-3α-one-17 in about the same amount as the androsterone. Dorfman (91) one year later also isolated etiocholanol-3α-one-17 from the urine of a castrated male treated with testosterone propionate.

To minimise the interference by endogenous steroids in the urine these studies were mostly carried out on hypogonadal males or women.

The administration of testosterone to a normal woman resulted in the excretion of the same metabolites with the possible addition of androstanediol-3α-17α(92). The latter compound was present in such small amounts that the investigators could not be sure it had arisen from testosterone metabolism.

Dorfman (93-94) was also able to isolate isoandrosterone from the urine of both a hypogonadal male and a guinea pig after the administration of testosterone propionate.

Table II summarizes the studies involved in the isolation of 17-ketosteroids after the administration of testosterone (95-96).

From these experimental data several workers postulated possible routes of testosterone metabolism (97-98-99). All of these schemes attempt to account for the conversion of testosterone to androsterone and its isomers. A total of twelve intermediate compounds are theoretically possible in this conversion.

To test these hypothetical pathways Dorfman (100-101) administered various ones of the postulated intermediates to hypogonadal males and isolated the urinary steroids resulting therefrom. After the administration of Δ^4androstenedione-3-17, increased amounts of both

TABLE II: 17-KETOSTEROIDS ISOLATED AFTER ADMINISTRATION OF TESTOSTERONE PROPRIONATE

SUBJECT	TESTOSTERONE PROPRIONATE ADMINISTERED MGM*	ROUTE OF ADMINISTRATION	17-KETOSTEROIDS ISOLATED IN % OF ADMINISTERED TESTOSTERONE				REF.
			2or3 $\Delta^{}$ androsterone-17	androsterone	isoandrosterone	etiocholanol-3α-one-17	
Male Hypogonad	501	oral	--	8%	--	--	90
Male Hypogonad	300	I.M.	--	9	--	--	90
Male Castrate	250	oral	--	6	--	--	91
Male Hypogonad	543	I.M.	--	11	--	10.7	89
Male Hypogonad	1002	I.M.	--	15	--	4.0	91
Rhesus Monkey	1002	subcu.	--	2.2	--	--	95
Male Guinea Pig	668	subcu.	--	--	2.4	--	94
Male Hypogonad	300	I.M.	--	--	11.7	--	93
Male Chimpanzee	2500	oral	4.7	2.1	--	0.8	96
Normal Female	1002	I.M.	3.4	14.6	--	10.1	92

*expressed as free testosterone

androsterone and etiocholanol-3α-one-17 were found in the urine. There was approximately twice as much of the latter compound. No isoandrosterone was demonstrated.

The administration of androstanedione-3-17 resulted in the isolation of androsterone and isoandrosterone from the urine but no etiocholanol-3α-one-17 was found.

When androstanediol-3α,17α was administered, androsterone was again isolated, along with small quantities of isoandrosterone. No etiocholanol-3α-one-17 was found.

Treatment with androsterone resulted in the finding of only androsterone in the urine. About 24% of the administered androsterone was accounted for.

In these studies by Dorfman the urinary androgens were measured simultaneously by bioassay. Although all the administered intermediates gave rise to a definite increase in the output of urinary androgens, it is interesting to note that only androsterone and Δ^4androstenedione 3,17 gave rise to as great an increase in androgenicity as testosterone propionate.

Because it has been isolated in large amounts from the urine of patients with adrenal cortical carcinoma and hyperplasia (56-57)and because it was not found to be increased in the urine of normal patients after the administration of testosterone, dehydroisoandrosterone, one of the most prominent androgenic C19 steroids in the urine, was thought to arise from the adrenal cortex. Mason, in his experiments on the secretions of the adrenal cortex, studied the metabolism of dehydroisoandrosterone, and used the same general technique as Dorfman. He was able

to isolate androsterone, etiocholanol 3α-one-17, Δ^5androstenediol-3β,17α, and unchanged dehydroisoandrosterone from the urine of Addisonian males and females and a man with panhypopituitarism after the administration of dehydroisoandrosterone (102-103). Thus it was established that a 3β 17-ketosteroid, dehydroisoandrosterone, probably arising from the adrenal, was also metabolised to the 3α-17-ketosteroids, androsterone and etiocholanol 3α-one-17, as well as testosterone. No studies have been conducted on the intermediates involved between dehydroisoandrosterone , androsterone, and etiocholanol 3α-one-17.

Through this type of in vivo study it was established that testosterone administration gave rise to increased amounts of androsterone, etiocholanol-3α-one-17, and possibly isoandrosterone. Δ^4androstenedione-3,17, androstanedione-3,17, androstanediol-3α,17α, and dehydroisoandrosterone, all of which are possible intermediates in the metabolism of testosterone to androsterone, have given rise to androsterone upon administration. The implication has been that these may be intermediates in the metabolism of testosterone.

Another approach to the problem of the metabolism of testosterone has been a study of the metabolic products formed after incubation of different tissues from experimental animals with testosterone. Studies have been extended to preparation and characterisation of the enzyme systems acting on the testosterone molecule.

The first to conduct studies of this nature with steroids was Zondek (104) in 1934. He showed that estrogens were inactivated by incubating with a liver brei.

Investigators in the androgen field soon recognised that the

liver might play a role in the metabolism of the androgens also. Darby (105) reported in 1940 that if a dog's liver or a cow's kidney is perfused with blood containing androgen, the latter is inactivated. She also incubated testosterone with pulped liver and kidney but reported no destruction.

Riskind et al (106) in 1940 also presented evidence that the liver inactivated testosterone. He demonstrated that castrated male rats with pellets of testosterone propionate implanted in their spleens would show castration effects of atrophy of the accessory sex organs as long as the spleen was left in situ. On the other hand, if the spleen with the implanted pellet were transplanted to the subcutaneous tissue or any other site in which the venous blood entered the general circulation by-passing the liver, the prostate and seminal vesicles would undergo enlargement.

By transplanting the animals' own testes to the mesentery Burrill and Greene (107) observed atrophy of the prostate and seminal vesicles, indicating that the liver could inactivate the animals' own testicular androgens.

The first definite demonstrations that liver tissue could metabolise testosterone when incubated in an isolated system were done independently by Kochakian and Samuels in 1944. Kochakian (108) et al showed that the incubation of testosterone with rabbit liver slices in serum gave rise to Δ^4androstenedione-3,17 and cis-testosterone. That this reaction was reversible was shown by the fact that similar incubations with Δ^4androstenedione-3,17 gave rise to testosterone (109). Samuels (110) also reported the enzymic destruction of testosterone by

rat livers in the same year.

In 1947 Samuels et al (111) reported a method of isolation and purification of the metabolites from tissue incubations with testosterone that permitted the accurate analysis of less than fifty µgms. of steroid. By a combination of photometric procedures, including both visual and ultraviolet spectral analysis, he was able to follow quantitatively various alterations in the testosterone molecule. Because of its relative simplicity and rapidity, large numbers of incubations under varying conditions could be run simultaneously. This rapid but accurate and sensitive method obviously was of great value in the characterization of the enzyme systems involved in testosterone metabolism and in the evaluation of various factors affecting these systems.

With these new experimental tools Samuels et al (112-113-114-115) were able to show that of a wide variety of tissues tested, including accessory sex organs in many different species of experimental animals, only the liver and kidney were active in the metabolism of testosterone.

Both organs exhibited a DPN-activated system which oxidized the 17-hydroxyl group in testosterone to a ketone. A cell free enzyme system, having only this activity as far as steroids were concerned, was prepared from steer liver (116). The end product of this system was identified as Δ^4androstenedione-3-17.

Another separate enzyme system in liver tissue was involved in the reduction of the α-β unsaturation of ring A and had citrate as its cofactor. No products were isolated. Only one species of animal

exhibited this activity as far as the kidney was concerned.

That other different enzyme systems involved in the reduction of the 17-ketone were present in liver tissue was recognized but these were not characterized.

Enzyme systems in the kidney were demonstrated that would oxidize a 17-hydroxyl group to a ketone under DPN activation in a number of different 17-hydroxyl steroids, namely testosterone, dihydrotestosterone, androstanediol-3α,17α, and androstanediol-3β,17α,(115). These are all possible intermediates in the metabolism of testosterone.

Conversely, a citrate activated system in kidney tissue reduced the 17-ketone group in Δ^4androstenedione-3,17, androsterone, androstanedione-3,17 and dehydroisoandrosterone (112).

Kochakian et al (117) have also shown that kidney tissue is active in the metabolism of testosterone. They succeeded in isolating Δ^4androstenedione-3,17 after incubating testosterone with kidney slices.

In their studies on the intermediary metabolism of steroids Schneider and Mason (113) incubated dehydroisoandrosterone with rabbit liver slices. They were able to isolate and identify Δ^5androstenediol-3α,17α, androstenetriol-3β,16β,17α, unchanged dehydroisoandrosterone, and cholesterol from the reaction mixture.

These same authors also incubated androsterone with rabbit liver slices and isolated androstanediol-3α,17α, androstanedione-3,17, isoandrosterone, and unchanged androsterone from the reaction mixture (119). Following similar incubations with etiocholanol 3α-one-17, the following steroid metabolites were isolated: etiocholanediol-3α,17α, etiocholanediol-3α,17β, etiocholanedione-3,17, and unchanged etiocholanol-3α-one-17.

It is significant to note that in their experiment a large part of the steroid metabolites were conjugated and had to be hydrolyzed for analysis. No study was made of the conjugate.

These incubation studies have established that both liver and kidney possess enzyme systems capable of altering the testosterone molecule. One might generalize by saying that whereas some enzymatic activities in the metabolism of testosterone have been better characterized than others, possible enzyme systems for nearly all the intermediate reactions in the metabolism of testosterone as postulated by Dorfman have been demonstrated directly or indirectly in either one or both liver and kidney tissue. A few reactions have not been studied. There are no reports on the incubation of any of the etiocholanediols. The products of the reduction of the A ring α-β unsaturation are also obscure. Although this reduction has been accomplished enzymatically with both testosterone and Δ^4androstenedione-3,17, it is not known whether the ketone or the double bond is reduced, or both.

Another phase of testosterone metabolism which must be considered is the form in which the steroid metabolites are excreted into the urine. The androgens in urine are water-soluble and biologically inactive. As early as 1929 Funk et al (120) were able to show that urine extracted after acidification gave larger amounts of active material than untreated urine. Adler (121) in 1934 showed that butanol extracts of male urine were inactive by capon comb test but could be converted to a biologically active material by heating with trichloroacetic acid. Koch et al (122-123) confirmed these findings. These studies established that the steroid androgen was excreted as a water-soluble "conjugate" that was biologically

inactive which on hydrolysis yielded a relatively water insoluble biologically active steroid.

The first "conjugated" steroid to be isolated from urine was K estriol glucuronidate in 1936 by Cohen and Marrian (124).

That same year Venning and Browne (125) succeeded in isolating Na pregnandiol glucuronidate from pregnancy urine.

In 1938 Schachter and Marrian (126) reported the isolation of K estrone sulphate from pooled urine of women.

The first conjugated androgen to be isolated was Na androsterone sulphate in 1942. It was obtained from the urine of a man suffering from interstitial cell carcinoma of the testis by Venning, Hoffman, and Browne (127).

Two years later Munson, Gallagher, and Koch (128) isolated Na dehydroisoandrosterone sulphate from normal male urine.

These two are the only conjugated androgens that have been isolated from urine. Both of these were ethereal sulphates. Evidence is available to indicate that urinary androgens are also conjugated with glucuronic acid. Koch et al (129) have reported a very marked correlation between the androgenic activity and glucuronic acid content on mens' urine extracts through different purification procedures. However, no androgenic glucuronides have been isolated.

Other non-androgenic conjugated steroids which have been isolated from human urine include: pregnanol-3α-one-20 glucuronide (130-131), pregnanetriol-3α,16,17 glucuronide (132), pregnanediol-3α,17-one-20 glucuronide (133), and Δ^{16}androstenol-3α glucuronide (134). In addition Δ^{16}allopregnenol-3β-one 20 sulphate, uranediol sulphate, and allopregnanol-

3β-one-20 sulphate have been isolated from mares' urine (135).

No investigations have been reported on the site of conjugation of steroids in the body. In 1939 Lipschutz and Bueding (136) demonstrated the formation of conjugated glucuronides when higher chain alcohols were incubated with kidney and liver slices. No steroids were used. Mason (118-119) observed "conjugation" in his steroid incubations with rabbit livers but no studies of the conjugates were made.

Deichman et al (137) on the other hand have evidence that the conjugation of glucuronic acid with cyclohexanone is not decreased in rabbits with moderate or severe liver damage induced by phosphorus or carbon tetrachloride administration. That the liver damage may not have been sufficient to interfere with the conjugation mechanism is indicated by the report of Lipschutz and Bueding (136) that fatty liver slices from guinea pigs poisoned with phosphorus still were able to form glucuronides in vitro, although at a diminished rate. Snapper and Saltzman (138) have noted that in patients with liver disease glucuronide conjugation of benzoic acid persists when the conjugation of glycine, a known liver function, has decreased.

Less is known about the site of formation of the ethereal sulphates.

Thus, by inferential reasoning, the liver is usually thought of as the site of conjugation of the steroids inasmuch as they also are higher alcohols and phenols. However, even with higher alcohols and phenols, other organs and tissues may be involved.

In the invivo studies involving testosterone administration and urinary isolations only a small percentage of the administered

testosterone could be accounted for as a steroid metabolite in the urine. The problem arises as to what has happened to the rest of the administered testosterone. That other routes of excretion, such as fecal, might be utilized has been indicated previously.

Another possibility is that non-steroidal metabolites which have not been analyzed have been formed.

That other steroid metabolites are formed through the metabolism of testosterone which have escaped detection is a distinct possibility. Evidence is available to indicate that androgens are converted to estrogens. Steinach et al (139) found that the administration of androsterone to castrated rats was followed by an increased excretion of estrogens in the urine by bioassay techniques. The same authors (140) reported that the same increase in estrogens occurred in men following the administration of either testosterone propionate or androsterone.

An increased output of estrogens in the urine, as measured by bioassay, has been observed by others and found definitely to be in the phenolic fraction of the urinary extract (141-142-143). No isolations have been reported.

Other steroid metabolites in the urine might have escaped detection.

In summary, it may be said that testosterone, the most potent of androgens, and the only one isolated from testis tissue, is considered to be the natural hormone secreted by the testis.

Urinary isolation studies have indicated that the final excretory products of testosterone metabolism in the intact animal and man are

androsterone and etiocholanol-3α-one-17, and possibly isoandrosterone and androstanediol 3α,17β. These steroid products account for less than 50% of the administered testosterone. The majority of the administered testosterone cannot be accounted for.

Studies on the intermediate metabolism of testosterone have shown that Δ^4androstenedione-3,17, androstanediol-3β,17α, and androstanedione-3,17 upon administration give rise to the same end products as testosterone administration. They are therefore considered intermediates in the metabolism of testosterone.

Incubation studies have indicated that the tissues of the body most involved in the metabolism of testosterone are the liver and kidney. Δ^4androstenedione-3,17 is the only definite product of these incubations, but enzymes capable of acting on nearly all possible intermediates have been demonstrated in either one or both of these organs.

Incubation studies have emphasized the importance of various cofactors in the route taken by testosterone metabolism.

Neither type of study has been able to establish which route is taken in the normal intact animal under normal physiological conditions.

It has been emphasized that the adrenal, ovary, and placenta can also give rise to androgenic steroids and, in the case of the adrenal, to some of the same androgenic steroids as those from testosterone metabolism.

All previous in vivo studies have been conducted with testosterone and derivatives of testosterone administered by some other route than the intravenous one. With the discovery of a method of intravenous administration of testosterone a study of the metabolism of intravenous testosterone was undertaken.

EXPERIMENTAL METHODS

I. THE PREPARATION OF TESTOSTERONE SOLUTIONS IN SERUM, PLASMA, AND SERUM ALBUMIN FOR INTRAVENOUS ADMINISTRATION.

Testosterone solutions for intravenous administration were prepared after the method of Bischoff (1). An excess of crystalline testosterone was added to a round bottom flask containing either serum, plasma, or 25% human serum albumin. The serum and plasma was prepared by bleeding experimental animals under sterile conditions just before preparation of the solutions. The 25% human serum albumin was obtained from the Red Cross.

The mixture of testosterone and albumin was then equilibrated by shaking in a Warburg apparatus at 37°C overnight.

After equilibration the excess undissolved testosterone was removed by filtration.

Aliquots of the filtered solutions were taken for testosterone assay. Quantitative analyses for testosterone were made on the ether extracts of these aliquots directly by the absorption at 240 mu in the ultraviolet range, as explained later.

To reduce the risk of bacterial contamination in the preparation of these solutions, bacteriostatic amounts of penicillin and streptomycin were added to the incubation media. The solutions were prepared the night before they were used. Sterile precautions were observed in all manipulations.

II. ANIMAL EXPERIMENTAL METHODS

Three animal species, the rat, rabbit, and dog, were used in

these intravenous administration studies. The testosterone dissolved in homologous serum or plasma was administered by way of the femoral vein from a syringe of suitable size. Periodic blood samples after administration were taken from the femoral artery and analyzed for their steroid content. At the end of the experiment, the animal was sacrificed and samples of various tissues taken for testosterone analyses. In some animals the effect of hepatectomy and nephrectomy upon the blood testosterone levels were studied. The kidneys were excised and the liver was functionally removed by ligating its entire arterial blood supply.

III. HUMAN EXPERIMENTAL METHODS.

Human subjects, both normal and diseased, were used in studies on the metabolism of intravenous testosterone. Testosterone dissolved in 25% human serum albumin was administered intravenously by means of a Baxter transfusion set. The 25% albumin solutions were given in 10 to 25 minutes. The half-way point of administration was taken as the experimental zero time. Blood and urine samples were taken periodically after administration and analyzed for their steroid content. The blood samples were oxalated to prevent clotting. Normal control samples were run on each subject.

IV. ANALYTICAL METHODS FOR STEROIDS IN THE BLOOD

A. "Free" steroids obtained from non-hydrolyzed blood.

Each blood sample, amounting to approximately 20 cc. in volume was hemolyzed by the addition of equal amounts of distilled water and extracted five times with equal volumes of redistilled ether. Ether extraction was done by agitating the aqueous ether mixtures in 100 cc. centrifuge tubes with footed stirring rods. Usually emulsions formed and had to be broken by centrifugation.

The supernatant ether was removed by siphoning under pressure. The methods of Samuels (111) were used for further purification.

The dried ether extracts were dissolved in 20 cc of hexane and chromatographed with a column of Baker and Adamson alumina 10 x 50 mm. The material was eluted with the following solvent fractions: (A) 30 mls. hexane, (B) 15 mls. of 10% chloroform in hexane, (C) 75 mls. of 25% chloroform in hexane, and (D) 75 mls. of chloroform. Testosterone and its metabolites were eluted quantitatively in the 25% chloroform in hexane fraction. This fraction was evaporated to dryness.

The dried 25% chloroform in hexane fractions were dissolved in 10 mls. of hexane and transferred quantitatively by rinsing with another 10 mls. of hexane. The hexane solutions were extracted by shaking first with 20 mls. of 70% ethanol and then with 15 mls. of 70% ethanol. The 70% ethanol extracts were combined and 15 mls. of distilled H_2O were added. The diluted ethanolic solutions were extracted three times with 15 mls. of redistilled chloroform. The combined chloroform extracts were evaporated to dryness under air jets.

Ethanolic solutions of the dried chloroform extracts were usually pure enough for steroid analysis. Occasional samples had to be rechromatographed and subjected to solvent partitioning again.

In order to determine the accuracy and sensitivity of this method for blood, model experiments were run in which testosterone in varying amounts was added to 20 cc of normal blood and processed in the above manner.

When 10 ugms. of testosterone were added to 20 cc of whole

blood, 82.5% to 107% of the testosterone could be recovered by this method in triplicate samples (Table II A). When larger amounts of testosterone (40γ and 576γ) were added, 97% to 104% was recovered.

Similar recovery experiments with androsterone demonstrated that as little as 5 ugms. of androsterone could be detected. The percentage of recovery from 12 samples containing varying amounts of androsterone from 5 to 25 ugms. averaged only 64.2%. (Table II B).

B. "Conjugated" steroids obtained from hydrolyzed blood.

Conjugated steroids in blood would not be ether extractable and would escape detection unless the blood were first hydrolyzed. No methods of hydrolysis for the isolation of steroids from blood were reported in the literature and a method had to be developed.

1. Method for the hydrolysis of conjugated steroids in blood.

The method involved hydrolysis of the conjugates followed by extraction of the free steroids with ether. Model experiments with sodium androsterone sulfate added to normal blood were run. Three micromoles of androsterone sulfate were added to 20 cc. of whole blood and the volume brought to 300 cc. with distilled H_2O. In order to determine the optimal conditions for hydrolysis concentrated HCl was added in percentage concentrations varying from 7 to 26 2/3% by volume. The time of hydrolysis was also varied. It

TABLE II A

Recovery of Testosterone Added

to Human Blood

Sample No.	Blood Volume cc.	Added Testosterone ugm.	Testosterone Recovered ugm.	% Recovery
1	20	40	40.2	101
2	20	40	40.6	102
3	20	40	38.8	97
4	20	576	598	104
6	20	576	569	98.6
7	20	10	10.7	107
8	20	10	10.2	102
9	20	10	8.25	82.5

TABLE II B

Recovery of Androsterone Added

to Human Blood

Sample No.	Blood Volume cc.	Added Androsterone ugm.	Androsterone Recovered ugm.	% Recovery
3	20	5	4.11	82.3
4	20	5	2.58	51.6
5	20	10	7.00	70.0
6	20	10	6.71	67.1
7	20	15	16.4	109
8	20	15	11.2	74.4
9	20	20	20.0	100
10	20	20	14.0	70.0
11	20	25	18.8	75.0
12	20	25	20.6	82.2

was also found necessary to use heat in order to hydrolyze the conjugates.

Hydrolysis for 15 minutes in 10% concentrated HCl at boiling temperatures gave the highest yields of free androsterone (Table III). An average of 51.8% of the theoretical amount of free androsterone released from the androsterone sulfate was recovered. Venning et al (127), in their hydrolytic studies with androsterone sulfate also observed poor recoveries of androsterone. Many other investigators have been aware of the fact that steroids underwent destruction during hydrolysis (144, 97). In urinary steroid isolation studies if a given method of hydrolysis gave reproducible results, it was considered adequate for quantitative analytical work. Hydrolysis of blood with 10% concentrated HCl at boiling temperature for 15 minutes was considered reproducible enough for use in analysis (See Table III).

Following hydrolysis, the reaction mixture was rapidly cooled to room temperature and transferred to a 2 liter separatory funnel. The mixture was extracted five times with 250 cc. volume of ether. Bad emulsions formed and had to be broken by centrifugation. The combined ether extracts were dark brown in color. They were washed with 2.5 N NaOH, 8N H_2SO_4, and distilled water. This procedure removed a good deal of the brown color

TABLE III

Methods of Hydrolysis of Blood for

17-ketosteroid Determinations

Exper. No.	No. of Indv. Hydrolyses	% HCl (by vol.)	Reflux Boiling Time	%androsterone SO_4 recovered*	Variation in % Recovery
1	1	7%	15	11.8%	
2	5	10%	15	51.8%	45.7-64.0%
3	1	13%	15	21.3%	
4	1	16 2/3%	15	18.6%	
5	2	20%	20	10.5%	9.8-11.2%
6	1	26 2/3%	15	28.0%	

* as free androsterone

from the ether extract, but it was still highly pigmented. Washing with 0.1 M Na_2HPO_4 or a saturated solution of $NaHCO_3$ removed practically all of the pigment without loss of steroid.

The colorless ether solution was then evaporated to dryness. This dried ether extract could not be used for 17-ketosteroid analysis by the Zimmermann reaction because of an interfering brown color that formed with the Zimmerman reagents. Further purification was effected by alumina chromatography and solvent partitioning, as described previously. With this purification, the steroid products were suitable for both 17-ketosteroid analysis by the Zimmerman reaction and α-β unsaturation analysis by the ultraviolet spectra.

2. A modification of Venning's method for extraction and analysis of conjugated steroids in blood.

In a personal communication (145), Dr. Venning reported the demonstration of 17-ketosteroids in the blood of a patient suffering with interstitial cell carcinoma of the testis. Her method of analysis utilized the fact that all known conjugated steroids are very soluble in ethanol as well as water.

A modification of her procedure has been developed for this study. To one volume of oxalated blood, four volumes of 95% ethanol was added to precipitate the blood proteins. The precipitated blood proteins were removed

by centrifugation and washed four times with 10 mls. of 95% ethanol. The ethanol washes were combined with the protein-free supernatant and the alcohol removed in vacuo.

The aqueous protein-free solution was diluted to 100 cc. with distilled water, hydrolyzed, extracted with ether, and purified for analysis by chromatography and solvent partitioning as described above. This procedure is easier technically because of the removal of the interfering proteins. Better yields of 17-ketosteroids were obtained with this method than with the previous method. When tested on the same post-injection blood sample, the first method gave an average of 278 ugms. % from three separate determinations; whereas the Venning method showed an average of 514 ugms. % (Table III A).

This modification of the Venning procedure was used in most of the present reported studies.

V. METHODS OF ANALYSIS FOR STEROIDS IN TISSUES

Aliquots of tissues were homogenized, transferred to 100 ml. centrifuge tubes, and extracted five times in the usual way with equal volumes of ether. The combined ether extracts were evaporated to dryness. The dried extract had to be partitioned between hexane and 70% ethanol before alumina chromatography. Otherwise the procedure was essentially the same as in the purification of the ether extracts from blood. The usual determinations were made for 17-ketosteroids and α-β unsaturated steroids.

TABLE III A

Comparison of Methods of Analysis for

Conjugated 17-ketosteroids in Blood

Sample No.	Method of Analysis	17-ketosteroids in mgm. %
1	Direct acid hydrolysis of blood	323
2	Direct acid hydrolysis of blood	227
3	Direct acid hydrolysis of blood	284
4	Acid hydrolysis of protein-free filtrate from blood (Venning Method)	441
5	Acid hydrolysis of protein-free filtrate from blood (Venning Method)	564
6	Acid hydrolysis of protein-free filtrate from blood (Venning Method)	537

To determine the percentage recovery of testosterone from fat by this procedure 576 ugms. of testosterone were added to 10 gms. of fat and analyzed in the usual manner. In duplicate samples the percentage recoveries were 94.5% and 91.9%.

VI. URINE ANALYSIS METHODS.

A. Routine urinary analyses.

Urine samples were first extracted four times with equal volumes of ether in a separatory funnel. These extracts contained the "free" steroids. The extracted urine was then hydrolyzed in the usual manner by boiling under reflux for 15 minutes in 10% concentrated HCl by volume.

The steroids liberated in this manner were designated the "conjugated" steroids. They were extracted from the reaction mixture with ether following hydrolysis. The ether extracts were washed with 2.5 N NaOH, 8N H_2SO_4, and distilled water. The ether extracts containing neutral steroids were evaporated to dryness.

In those studies in which only 17-ketosteroid analyses by the Zimmerman reaction were needed, no further purification was necessary. For $\alpha\beta$ unsaturated steroid determinations, it was necessary to purify by usual methods of alumina chromatography and solvent partitioning, as described above.

B. Isolation and identification of "free" urinary steroids.

Isolation and identification studies were carried out on the "free" neutral steroids from several pooled post-injection urines as obtained above. Several different procedures were used in the purification of these steroids for identification.

1. Girard separation: The ketonic and non-ketonic constituents of the "crude neutral fraction" were separated by means of the Girard's Reagent T (146). This reagent, trimethylaminoacetohydroside hydrochloride, reacts with ketones forming the water soluble chloride salt derivative. Extraction with ether removes the non-ketones, leaving the Girard T ketone derivative in water solution. The ketonic steroids are recovered quantitatively by ether extraction after hydrolysis of the Girard T derivative.

Many suitable modifications of the Girard separation exist. For the exact method used in this study, refer to Experimental Results.

2. Digitonin separation: Steroid compounds containing a free 3β hydroxyl group will react with digitonin to form a digitonide which is insoluble in 90% ethanol, whereas steroids with a 3α hydroxyl group do not. (147) By virtue of this fact, these two important groups of steroids can be separated.

Again many suitable methods of digitonin separation exist. In this study the procedure of Butler and Marrian (148) was used with minor modifications. For the exact technique used, see Experimental Results.

3. Separation of alcoholic and non-alcoholic non-ketones with succinic anhydride: (149) The principle of this separation is that alcoholic steroids will react with succinic anhydride to form a hemisuccinate. The hemisuccinate, having a free acid group, is removed from an ether solution by alkaline washes whereas neutral non-alcoholic steroids stay

in solution. Refer to Experimental Results for exact procedure used in this study.

4. Chromatographic separations. (150)

Chromatography on Merck alumina was used to separate both ketones and non-ketones. The ratio of alumina used to steroid chromatographed was 30:1 in all cases. Various solvent mixtures were used in developing the columns. For the ketones hexane and mixtures of benzene in hexane were used. In some instances it was necessary to elute with ether in benzene and pure ether. See Experimental Results for actual data.

5. Technicon chromatographic separation.

By repeated chromatographies as reported above, it was possible to separate all but 14.1 mgms of α-ketonic steroids. By chromatographing these 14.1 mgms. on Baker and Adamson alumina using the automatic Technicon fraction collector eluting with 10% chloroform in hexane, it was possible to identify all but 2.6 mgms. of this mixture. A 60 x 10 mm. column of alumina was used and 65 fractions of 2.0 cc each, 25 fractions of 5.0 cc each, and 18 fractions of 10.0 cc each were collected. For further details see Experimental Results.

6. Acetylation procedures.(150)

Acetylation was used as a method of purification of the steroids. By virtue of the fact that acetates have different adsorptive properties than the parent steroid compound separation by chromatographing the acetates can often be

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effected. See Experimental Results for details.

7. Sublimation in high vacuum at temperatures ranging up to 180° C was used in the purification of both free steroids and their acetates.

8. Identifications of crystalline products were made by melting point determinations, mixed melting points, preparation of derivatives, and absorption spectra in both the infra-red and ultra-violet range. All melting points were taken on the kofler melting point apparatus. Infra-red spectra were made on the Beckman spectrophotometer. Identification was made by comparison of the absorption of the unknown in the so-called "fingerprint" region from 1185-875 cm.-1 to that of known standards. Infra-red spectra were run on only those fractions which could not be identified absolutely by other usual means. These were usually fractions which could not be crystallized.

C. Isolation procedure for a conjugated 17-ketosteroid from urine.

Aliquots of urine from 2 normal male subjects collected for 4 hours after the injection of testosterone were examined for their "conjugated" 17-ketosteroids. The urines were extracted five times with n-butanol at pH1.0. Butanol extracts were evaporated to dryness in vacuo and worked up separately.

Alumina chromatography was used in both purifications. Both Baker and Adamson and Merck alumina were used in a ratio of 30 parts alumina to 1 part material. The eluent solvents consisted of increasing amounts of ethanol in acetone.

Chromatography with powdered starch was also used. The starch was washed well with water before use. The column was developed with water-saturated butanol and water.

A technique of paper chromatography was developed as an aid in the identification of water-soluble conjugated 17-keto-steroids in urine. Both ascending and descending types of columns were used. 25 to 100 ugms. of the conjugated 17-ketosteroids in either a water or alcohol solution were applied to Whatman No. 2 filter paper by means of a capillary pipette. Care had to be taken in the application that the spot did not exceed 0.5 cm. in diameter. The paper chromatogram was placed then in an air-tight glass jar 24 x 12 inches and developed with H_2O saturated n-butanol for 48-72 hours or until the solvent front had traveled from 30-40 cm. The paper chromatogram was then removed and dried at room temperature.

The dry chromatogram was sprayed with an atomizer containing 2 parts of a 1% ethanolic solution of metadinitrobenzene and 1 part of 2.5 N ethanolic KOH. Upon standing in an atmosphere of ethanol for 20-40 minutes, the 17-ketosteroids became visible as distinct purple spots. The color was stable for from 1 to 2 weeks after which it faded to a faint brown.

To test the specificity of this color reaction on filter paper, the following steroids were spotted on filter paper and sprayed with the m-dinitrobenzene solution: androsterone, androsterone sulfate, estrone sulfate, testosterone, dehydroisoandrosterone, Δandrostenedione-3,17, androstanedione-3,17, desoxycorticosterone, methyl testosterone, and progesterone. Only those steroids having a

17-ketone developed a purple color. The other steroids gave either no color or an immediate brown color that became a bluish-black in a few hours.

For details on the isolation of conjugated 17-ketosteroids, see Experimental Results.

VII. QUANTITATIVE ANALYTICAL METHODS.

A. Testosterone determinations.

Steroids with an α-β unsaturated ketone in their structure absorb light in the ultraviolet end of the spectrum with a maximum absorption at 240 mu. in a solution of ethanol. The light absorption at 240 mu. is proportional to the concentration of the α-β unsaturated steroid and can be used in quantitative measurements.

In actual practice solutions containing about 40 ugms. of unsaturated steroid in 3 cc. of ethanol were read on the Beckman spectrophotometer from 220 to 300 mu. at 10 mu. intervals. Standard solutions, tissue blanks, and reagent blanks subjected to the same purification procedures, were similarly analyzed.

Two types of mathematical analysis of the absorption data were used. One method requires that the absorption of light by all contaminating materials in the sample must follow a straight line in the region in which the substance analyzed has its maximum absorption. Under these conditions, it can be proved that the sum of the optical densities at two wave-lengths equidistant from and on either side of the wave-length of maximum absorption of the compound to be measured will be equal to twice the optical density at the wave-length of maximum absorption if there is no compound to absorb. The difference between

twice the optical density at the wave-length of maximum absorption and the sum of the optical densities at the two equidistant wave-lengths is therefore proportional to the concentration of the substance measured. By comparison to a known standard concentration quantitative measurements can be made.

In the second method the absorption curve of the unknown is corrected by subtracting the absorption of the blanks. By comparison to a standard curve at several wave-lengths ratios between the absorption of the unknown and standard are determined. These ratios are constant if the proper corrections for background have been made and directly proportional to the ratio of the concentration of the unknown solution to that of the known standard. From this equation the concentration of the unknown can be determined.

Testosterone, having an $\alpha-\beta$ unsaturated ketone in ring A, can be measured quantitatively in this manner. It should be noted that this method is not specific for testosterone as any steroid having an $\alpha-\beta$ unsaturated structure will also absorb light in this region.

B. Determination of 17-ketosteroids.

Zimmerman (12) first observed that ketosteroids react with an alkaline solution of m-dinitrobenzene to give a reddish-purple color and suggested its use as a method of analysis.

Callow, et.al. (14) expanded upon Zimmerman's findings and established that 17-ketosteroids reacted with alkaline m-dinitrobenzene to give a purple color absorbing maximally at 510-520 mμ. and could be used in the quantitative estimation of 17-ketosteroids.

The Callow modification of the Zimmerman reaction was used in

this study. 50-100 mgms. of 17-ketosteroids were dissolved in 0.2 cc. of ethanol. 0.2 cc. of a 1% solution of m-dinitrobenzene in ethanol and 0.2 cc. of 2.5N KOH in ethanol were added.

The tubes prepared in the above manner were stoppered and incubated at 25°C for 90 minutes in the dark. 10 mls. of absolute alcohol were then added to each tube and the absorption read on a Coleman universal spectrophotometer from 440 mu. to 620 mu. at 20 mu. intervals. By comparison to the absorption of a standard solution of androsterone run simultaneously, the amount of unknown 17-ketosteroid was determined.

C. Glucuronic acid determinations.

Glucuronic acid determinations were made by the method of Maughn, et al (151). This method is a quantitative colorimetric analysis utilizing the violet color produced by the reaction of glucuronic acid with napthoresorcinol as originally described by Tollens (152). Maughn, et al showed that under the conditions of their procedure this color development obeyed the Beer-Lambert law. Accurate measurements of as little as 40 ugms. could be made. They also showed that the procedure was adaptable to urine.

2 cc. of a solution containing a 40-100 ugms. of glucuronic acid, 2 cc. of a 0.2 solution of napthoresorcinol in water, and 2 cc. of concentrated HCl were measured into suitable test tubes and placed on a boiling water bath for 30 minutes. The tubes were then removed and placed in an ice bath for 5-10 minutes. The contents of the tubes were rinsed into individual separatory funnels with 2 cc. of 94% ethanol and 5 cc. of redistilled anhydrous ether washed with a 1% ferrous sulfate solution. The colored ether layer was

removed and the aqueous solution extracted again with ether. The ether extractions were combined and the final volume adjusted to 10 ml. in a graduated colorimetric test tube. The tube was stoppered to prevent evaporation of the ether and the absorption read on a Coleman universal spectrophotometer from 500-640 mu. Maximal absorption was at 570 mu. Glucuronic acid standards and blanks were run simultaneously. Quantitative calculations from the absorption data were made in the usual manner.

EXPERIMENTAL RESULTS

Studies on Circulating Testosterone in Experimental Animals

The rate of disappearance of intravenous testosterone from the circulation of two intact dogs and three intact rabbits is indicated in Fig. 1. In this figure the percentage of the "original blood level" of testosterone is plotted against time. The "original blood level" is that concentration of testosterone to be expected in the blood if it did not disappear from the circulation. By estimating the blood volume from the total body weight and knowing the amount of testosterone administered, this theoretical concentration of testosterone in the blood can be closely approximated.

It is apparent that testosterone administered intravenously disappeared very rapidly from the blood. In all four animals 90% or more of the testosterone had left the circulation within 10 minutes after administration. The process appears to be biphasic, the remaining 10% disappearing at a much slower rate relative to concentration.

Effect of the Liver and Kidney on the Metabolism of Circulating Testosterone in Experimental Animals

Previous investigators have demonstrated by *in vitro* experiments that the liver and kidney metabolize testosterone (*10*, *11*, *12*). To determine what role these organs might play in the disappearance of testosterone from the circulation, testosterone was injected into an intact anesthetized animal and periodic blood samples analyzed for testosterone. The same animal was then hepatectomized, testosterone again administered, and blood samples again taken periodically. The procedure was repeated for a third time after the same animal had also been nephrectomized.

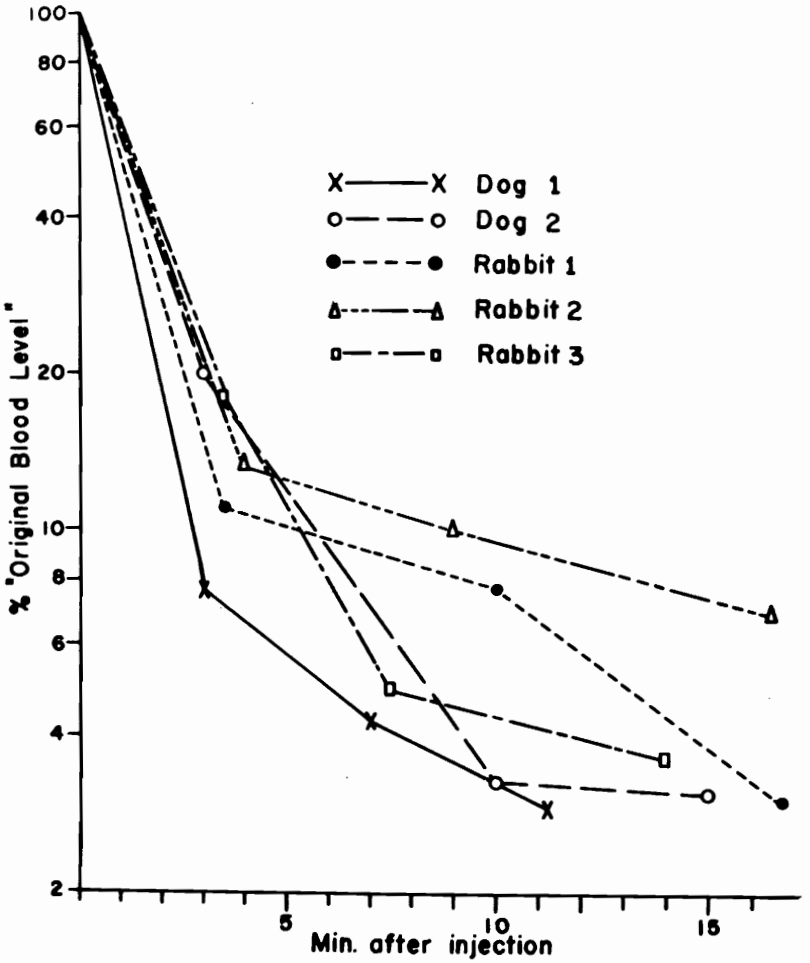

100
80
60
40
20
10
8
6
4
2
% "Original Blood Level"
X——X Dog 1
o——o Dog 2
●----● Rabbit 1
Δ----Δ Rabbit 2
▫----▫ Rabbit 3
5
10
15
Min. after injection

Fig. 1

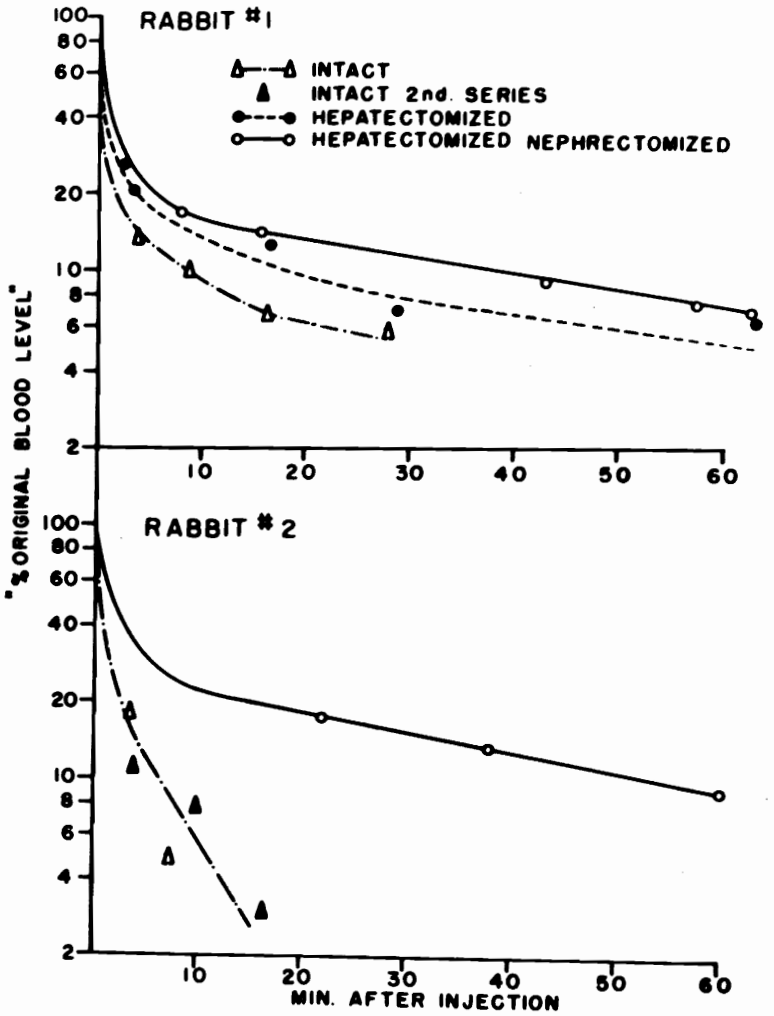

RABBIT #1
Δ—Δ INTACT
▲ INTACT 2nd. SERIES
●---● HEPATECTOMIZED
o—o HEPATECTOMIZED NEPHRECTOMIZED
RABBIT # 2
"% ORIGINAL BLOOD LEVEL"
100
80
60
40
20
10
8
6
4
2
10
20
30
40
50
60
MIN. AFTER INJECTION

Fig. 2

Experiments of this type were done in three dogs and two rabbits. The results in two typical experiments are indicated in Fig. 2. Neither removal of the liver alone nor the elimination of the kidneys in addition affected the rapid rate at which the testosterone disappeared during the first phase. During the second phase, however, the elimination of each organ decreased the rate of disappearance.

Analysis of Tissues for Testosterone after Intravenous Administration in the Experimental Animal

In all the experiments, 75% or more of the testosterone had disappeared from the circulation within ten minutes after injection, even in an hepatectomized, nephrectomized animal. Obviously the liver and kidney were not involved in this loss and it became necessary to look for other explanations.

One possible explanation was that the testosterone had diffused out of the circulation into the tissues. To check this possibility, rabbits were injected intravenously with testosterone and sacrificed one hour after injection. Samples of various tissues were removed at that time and analyzed for their testosterone content. The results of these analyses are summarized in Table IV.

All tissues tested, with the exception of liver and prostate, contained appreciable amounts of testosterone. The tissues, in order of decreasing concentration, were: fat, lung, spleen, kidney, heart muscle, skeletal muscle, and testis. Fat had by far the highest concentration. Identical tissues from normal un-injected male rabbits were analyzed simultaneously as controls and were negative for α-β unsaturated ketosteroids. The testosterone was present in the tissues in the free form; it was readily extractable with ether and hydrolysis

TABLE IV: TISSUE TESTOSTERONE CONTENTS
FOLLOWING INTRAVENOUS ADMINISTRATION IN RABBITS

RABBIT NUMBER	BODY WEIGHT	MGMS TESTO-STERONE I.V.	TISSUE	TOTAL BODY TISSUE GMS.	TISSUE TESTOSTERONE CONCENTRATION UGM./100 GMS.	MGMS. TESTOSTERONE IN TOTAL BODY TISSUE
1	3.9 kilo	21.5	fat	364.0	1140	4.16
			skeletal muscle	1720.0	204	3.51
			testes	4.9	130	0.01
			heart muscle	9.3	350	0.03
			lung	23.4	458	0.11
			spleen	1.1	452	0.01
			blood	258.0	448	1.26
			kidney	10.1	370	0.04
			liver	195.0	0	0
			prostate	1.5	0	0
			Totals	2587.3		9.13

TABLE IV (CONTINUED)

RABBIT NUMBER	BODY WEIGHT	MGMS TESTO-STERONE I.V.	TISSUE	TOTAL BODY TISSUE GMS.	TISSUE TESTOSTERONE CONCENTRATION UGM./100 GMS.	MGMS. TESTOSTERONE IN TOTAL BODY TISSUE
2	2.9 kilo	21.5	fat	270.0	2140	5.77
			heart muscle	5.7	724	0.04
			skeletal muscle	1320.0	222	2.94
			blood	192.0	597	1.15
			Totals	1787.7		9.90

did not yield additional testosterone.

There were no 17-ketosteroids by the Zimmermann reaction in the extracts of either unhydrolysed or hydrolysed tissues or blood. Neither Δ-βunsaturated ketosteroids nor 17-ketosteroids could be demonstrated in the urine passed by these animals during the experimental period.

An attempt was made to determine the proportion of the total amount of testosterone administered which was present in the various tissues studied (Table IV). This was calculated from the testosterone concentration in the given tissue and the total weight of the tissue in the whole animal. In the case of organs such as heart, spleen, liver, kidney, etc., the tissue weights were obtained directly; in the case of such tissues as fat, skeletal muscle, and blood, direct weighing was impossible, and certain approximations had to be made from data on body composition in the literature.

In Rabbit No. 1 42.5% of the administered testosterone could be accounted for in an analysis of tissues making up 67% of the total body weight. In Rabbit No. 2, an hepatectomized, nephrectomized animal, only 46% of the injected testosterone was accounted for in tissues making up 65.5% of the hepatectomized, nephrectomized carcass weight. (Table IV) These tissues accounted for 41.7% of the hormone in the intact rabbit. It does not seem, therefore, that removal of the liver and kidneys greatly affected the distribution of testosterone among the tissues during the first hour after injection.

To determine how long testosterone stayed in the fatty tissues after intravenous injection, the following experiment was run. Eighteen

rats were divided into three groups of six rats each. Rats in Group 1 served as controls; each received 0.6 ml. serum intravenously. The rats in Group 2 were each injected with 0.822 mg. of testosterone dissolved in 0.6 ml. of serum. These groups were sacrificed one hour after injection. Rats in Group 3 received the same treatment as those in Group 2, except that they were sacrificed three hours after injection. Samples of fat were taken from each rat and pooled within groups for determination of testosterone.

The samples of fat from the controls (Group 1) contained no demonstrable testosterone. There was a concentration of 913 ug. testosterone per 100 gm. fat in the animals sacrificed one hour after injection with the steroid (Group 2), but no measurable amount in the fat of those rats sacrificed three hours after injection (Group 3). Thus it would appear that testosterone stays in the fat stores for only a short period of time after intravenous administration.

Metabolism of Intravenous Testosterone by Human Subjects

Experiments with intravenous testosterone in human subjects were next undertaken. The first problem was to find a suitable solvent for the testosterone in which as much testosterone could be administered in as little volume as possible, both to make steroid determinations possible and to reduce the risk to the subject.

The Solubility of Testosterone in 25% Human Serum Albumin

Bischoff et al (1) had shown that the increased solubility of testosterone in serum was principally due to the albumin fraction. The

solubility of testosterone in the 25% human serum albumin was measured to determine its suitability for use. It was found that 2.31 mgm. of testosterone per ml. or 80.2×10^{-4} moles per liter could be dissolved in 25% human serum albumin. Bischoff had found that 14.2×10^{-4} moles of testosterone could be dissolved in one liter of 3% bovine albumin.

With this concentration of testosterone in 25% human serum albumin, it was theoretically possible to administer 200 mgms. of testosterone intravenously without danger to the subject. The theoretical expected blood concentration of testosterone in a 70 kilo. man after the administration of 200 mgms. was calculated to be 3.08 mgm. per 100 ml. of blood, on the basis of a total blood volume of 6500 cc. This concentration of testosterone was within the limits of analysis.

Precautions in the Administration of Albumin-Testosterone Solution to Human Subjects

Each patient receiving intravenous testosterone had a complete physical examination with special attention paid to the heart to avoid cardiac complications which might result from overloading the circulation with albumin. The first patient to receive intravenous testosterone was a terminal case of carcinoma. 200 mgms. of testosterone dissolved in 100 cc. of 25% human serum albumin were administered in ten minutes with no ill effects.

Since the first patient, testosterone in 25% albumin has been administered to a total of twenty-eight human subjects, both normal and diseased. There have been pyrogen reactions in two cases, lasting from thirty to sixty minutes, with urinary suppression lasting a maximum of one hour. One of these pyrogen reactions was thought to be due to improper cleansing and sterilization of the transfusion set. The second

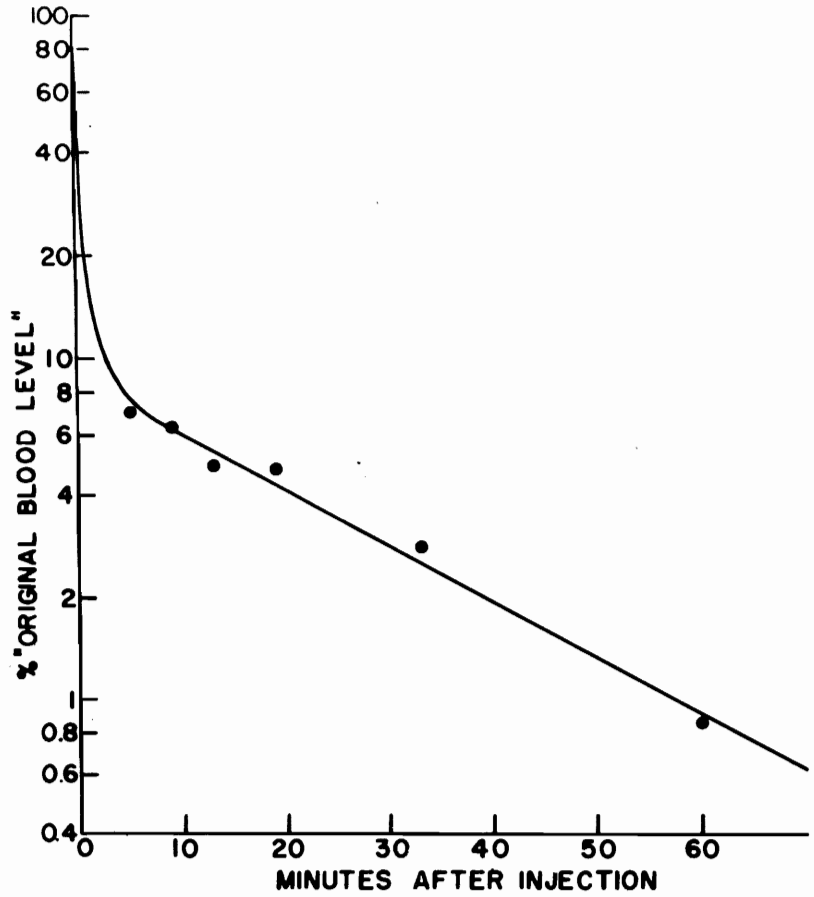
reaction was thought to be due to bacterial contamination of the albumin solution during preparation.

One case that had already received intravenous testosterone once and was to receive it again had a positive skin reaction to an intracutaneous injection of the solution prior to the second injection. No studies have been made on the immune reactions to solutions of testosterone serum albumin. This one case does indicate, however, that subjects receiving the material may build up an immunity to some component of the testosterone-albumin complex. Careful skin and opthalmic tests for allergy were run on all subjects.

No other reactions to intravenous testosterone in 25% human serum albumin were observed.

Studies on Circulating Testosterone in Human Subjects

As in experimental animals, testosterone administered intravenously to human subjects disappeared very rapidly from the circulation. In Fig. 3 the percent of the "original blood concentration" of testosterone in the blood of subject CE is plotted against time after injection. The "original blood concentration" is that theoretical concentration of testosterone that would be found if none of the testosterone were lost from the circulation or destroyed. It is calculated from the estimated blood volume and amount of testosterone given. Only 7.5% of the expected concentration could be demonstrated five minutes after administration. The decline of testosterone concentration in the blood followed a


100
80
60
40
20
10
8
6
4
2
1
0.8
0.6
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% "ORIGINAL BLOOD LEVEL"
0
10
20
30
40
50
60
MINUTES AFTER INJECTION

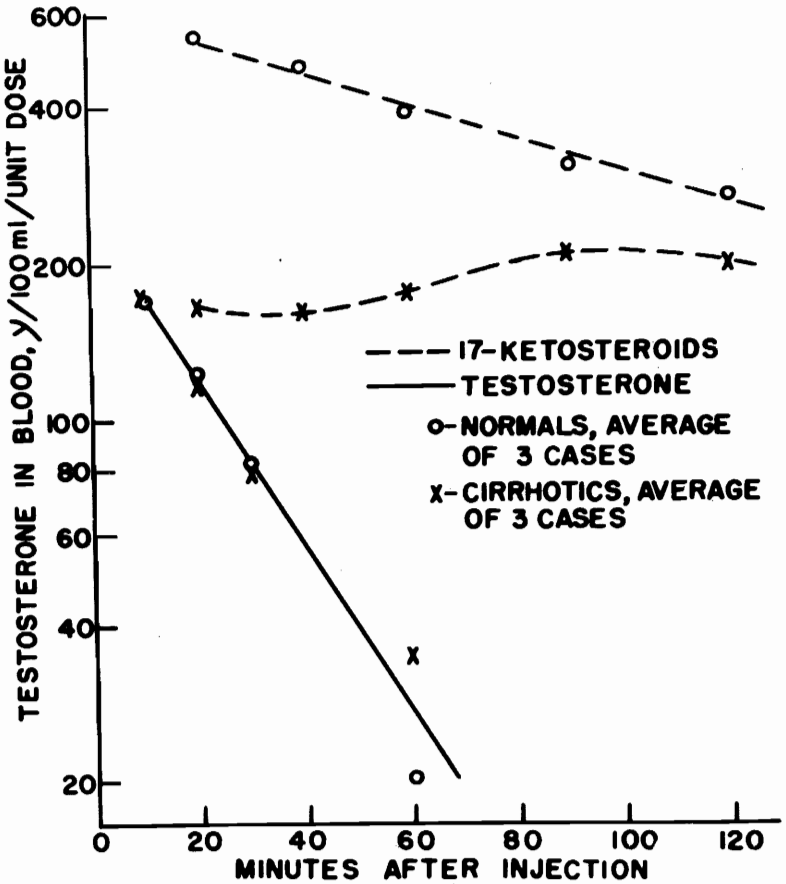

600
400
200
100
80
60
40
20
TESTOSTERONE IN BLOOD, y/100 ml/UNIT DOSE
17-KETOSTEROIDS
TESTOSTERONE
o-NORMALS, AVERAGE OF 3 CASES
X-CIRRHOTICS, AVERAGE OF 3 CASES
0
20
40
60
80
100
120
MINUTES AFTER INJECTION

Fig. 3

logarithmic course after the first rapid loss of testosterone. By sixty minutes after the injection the blood concentration had fallen to a level which was just within the range of analysis (Table V).

Although the series of cases in this study is not large, the results indicate that the various diseased states had little influence on the rate of disappearance of circulating testosterone. In each case less than 10% of the expected blood level, had none of the testosterone injected been metabolized or lost from the circulation, was found ten minutes after administration (Table V).

Blood 17-ketosteroids after Intravenous Testosterone in Human Subjects

No measurable amounts of 17-ketosteroids could be detected in the extracts of unhydrolyzed blood samples taken after the administration of testosterone. However, when these samples were hydrolyzed 17-ketosteroids were demonstrable. Samples of blood from normal subjects before the injection of the hormone had no demonstrable 17-ketosteroids by this method, either before or after hydrolysis.

In order to determine what portion of the whole blood carried the 17-ketosteroids, 400 ml. of oxalated blood were collected from a normal subject fifty minutes after testosterone administration. From this single sample 17-ketosteroid analyses were run in triplicate on both plasma and whole blood. When corrections for the hematocrit were made on the values for whole blood, it was

TABLE V: TESTOSTERONE BLOOD LEVELS FOLLOWING INTRAVENOUS ADMINISTRATION IN NORMAL AND DISEASED HUMAN SUBJECTS

PATIENT NO.	DIAGNOSIS	UGM. OF TESTOSTERONE PER 100 MLS. OF BLOOD PER UNIT DOSE*			
		10 MIN.	20 MIN.	30 MIN.	60 MIN.
CE	Normal	158	107.0	70.8	20.8
CN	Normal	153	128.0	102.0	27.6
CR	Normal	--	199.0	30.6	0
CG	Cirrhosis	187	112.0	57.0	20.6
CK	Cirrhosis	158	86.0	53.3	51.0
CL	Cirrhosis	182	152.0	123.0	33.9
CW	Nephritis	--	--	159.0	76.2
CZ	Nephritis	--	--	57.1	22.6
CC	Carcinomatosis	215	135.0	76.7	0
DA	Carcinomatosis	--	--	116.0	41.3
CX	Male Castrate	--	--	84.4	15.6

* = unit dose = 1 mgm. of testosterone per lb. of body weight

found that at least 96% of the 17-ketosteroids were in the plasma and no more than 4% were associated with the red cells and other formed elements of the blood. It is highly probable that all of the conjugated steroid is in the plasma.

Table VI lists the levels of conjugated 17-ketosteroids found in plasma at various intervals after the injection of testosterone. Seven normal patients, four patients with liver disease, and one patient with kidney disease were included. From these results it is apparent that the administered testosterone was very rapidly metabolized to conjugated 17-ketosteroids in the normal subjects. In the majority of the cases the earliest specimens, twenty minutes after injection, had the highest 17-ketosteroid concentrations. When these plasma concentrations are plotted against time (Fig. 4), it would appear from the shape of the curves that the maximum concentration of 17-ketosteroids in the blood occurred even earlier than twenty minutes after injection. The rate of fall thereafter was proportionate to the concentration.

The total amount of 17-ketosteroids in the circulation can be roughly approximated from their concentration in blood and the total blood volume assuming that plasma volume is equal to 5% of the body weight. The average total amount of 17-ketosteroids in the plasma of four normal subjects receiving an average dose of 167 mgms. of testosterone was 18.9 mgms. twenty minutes after the injection. This represented 11% of the injected testosterone. It was probable that more had been metabolized to

TABLE VI

Conjugated 17-ketosteroid Plasma Levels Following Intravenous Testosterone in Normal and Diseased Human Subjects

Patient No.	Diagnosis	Mgm. of 17-ketosteroids* per 100 ml of plasma per unit dose ₮					
		20 min.	40 min.	60 min.	1½ hr.	2 hrs.	3 hrs.
D K	Normal			463			
D L	Normal	726	630	540	437	360	238
D Q	Normal	348	403	349	329	300	225
D S	Normal	576	479	382	277	240	170
C R	Normal		445				
C H	Normal		475				
D T	Normal	525	390	278	177	155	107
D M	Hepatitis	180	122	108	166	160	182
D O	Cirrhosis	181	218	251	302		
D R	Cirrhosis	141	146	163	158	149	
C K	Cirrhosis			24			
C Z	Nephritis			812			

* As androsterone

₮ Unit dose = 1 mgm. of testosterone per lb. of body weight

17-ketosteroids and either excreted into the urine or stored in tissues.

The Effect of Liver and Kidney Disease on the Blood 17-ketosteroid Concentrations after Intravenous Testosterone

The concentrations of 17-ketosteroids in plasma after testosterone was injected intravenously into normal subjects were much higher than in patients with liver disease (Table VI). At twenty minutes after injection the average concentration in four normal subjects was 544 ugms. per 100 ml. of plasma; whereas, in three cases of liver disease the concentration was 167 ugms. per 100 ml. The concentration in the blood of these patients did not decrease logarithmically with time as in the normals, but actually increased during the first ninety minutes (Fig. 4). Various clinical tests showed that all these patients had impaired liver function. Although only a small number of patients was used, this marked difference indicates that the ability to metabolize testosterone to 17-ketosteroids has been altered in patients with liver disease.

The highest concentration of 17-ketosteroids in plasma was obtained in a case of severe glomerulonephritis. At one hour after injection the plasma level was 812 ugms. per 100 ml. of plasma, as compared to an average value of 402 ugms. per 100 ml. in the normal subjects. An endogenous creatinine clearance run simultaneously with this experiment showed that only 13.5 ml. of plasma were cleared per minute. Apparently the elevated plasma 17-ketosteroid level was principally the result of

this reduction in glomerular filtration.

Urine α-β Steroid Content after Intravenous Testosterone

In these experiments the urines also were examined for their steroid content. Non-hydrolyzed urines were first extracted with ether and then hydrolyzed in the usual manner and again extracted with ether. In this manner the "free" steroids were separated from the "conjugated" steroids.

Table VII shows α-β unsaturated steroid content of urines after intravenous testosterone administration in five normal subjects, two nephritics, and one male castrate.

These results indicate that after intravenous testosterone an unsaturated compound was excreted into the urine, which was not present prior to administration. Presumably this α-β unsaturated steroid was testosterone, but it may have been any other α-β unsaturated steroid resulting from testosterone metabolism.

Considering the dose of testosterone administered, only a small amount (less than 2%) was excreted into the urine.

From the data on concentrations of testosterone in the blood and urine, calculations of the renal clearance of testosterone were made by the method of Dominguez, Corcoran, and Page (154). It was found that less than 2 cc. of plasma were cleared of testosterone per minute. This low clearance may have been due to either a lack of filtration or a high renal threshold with almost complete tubular reabsorption.

"Free" 17-ketosteroids in the Urine after Intravenous Testosterone in Human Subjects

Small but definite amounts of free 17-ketosteroids were demonstrable in the ether extracts of non-hydrolyzed urines following

TABLE VII

Free and Conjugated - Unsaturated Steroids

in the Urine after Intravenous Testosterone

Patient No.	Diagnosis	Mgm. Testosterone I.V.	Urine - Unsaturated Steroids in Mgms. 1st hour free	1st hour conj.	2nd hour free	2nd hour conj.	3rd hour free	3rd hour conj.	4th hour free	4th hour conj.	5th hour free	5th hour conj.
CE	Normal	162	107	1100	40	513	10	202	0	91		0
CN	Normal	147	20	1051	4	577	0					
CR	Normal	168	185	1306	20	566	0					
CV	Normal	165	23	602	3	478	0	373		206		
CH	Normal	188				515		14		0		0
CW	Nephritis	169	49	552	0	595	0	321				
CZ	Nephritis	164	0	0	0	0						
CX	Male Castrate	166	101	1317	0							

intravenous testosterone that were not present in normal urines (Table VIII).

Conjugated 17-ketosteroids in the Urine after Intravenous Testosterone in Human Subjects

Large amounts of conjugated 17-ketosteroids were demonstrated in the ether extracts of hydrolyzed urines of subjects receiving intravenous testosterone.

Three subjects were studied for their urinary 17-ketosteroid excretion before and after testosterone administration. (Table IX)

In normal patient CE 71.5% of the administered testosterone was excreted as conjugated 17-ketosteroids into the urine during the first twenty-four hours after injection. During the post-injection period another 2.6% was excreted, accounting for a total of 74.1% of the testosterone.

In normal patient CH these figures were 53.9% in the first twenty-four hours and 18.3% in the post-injection period, making a total of 72.2%. In the carcinomatous patient CC 53.5% was excreted in the first twenty-four hours and 22.2% in the post-injection period, making a total of 75.7%. Another normal subject, not considered previously, excreted 73.4% of the administered testosterone in twenty-four hours as conjugated 17-ketosteroids.

In these three subjects an average of 74% of the injected testosterone was accounted for by an increase in urinary conjugated 17-ketosteroids. An average of 63.1% was excreted the first twenty-four hours after injection.

Hourly Urinary Conjugated 17-ketosteroids after Intravenous Testosterone in Human Subjects

Hourly urines were analyzed after the administration of

TABLE VIII: FREE 17-KETOSTEROIDS IN URINES AFTER INTRAVENOUS TESTOSTERONE

PATIENT NO.	DIAGNOSIS	MGMS OF TESTO-STERONE I.V.	URINE 17-KETOSTEROIDS IN UGM.		
			1st HR.	2nd HR.	3rd HR.
CN	Normal		172	35	0
CR	Normal		185	47	0
CV	Normal		24	5	0
CW	Nephritis		0	0	0
CZ	Nephritis		42	21	0
CX	Male Castrate		119	-	-

TABLE IX

Urinary Excretion of Conjugated 17-ketosteroids

Following Intravenous Testosterone

Normal Patient CE		Normal Patient CH		Ca. Patient CC	
Exper. Day	17 ketos. mgm/24 hr.	Exper. Day	17 ketos. mgm/24 hr.	Exper. Day	17 ketos. mgm/24 hr.
1	11.2	1	7.3	1	17.6
2	13.6	2	8.8	2	25.7
3	11.2	3	9.9	3	12.1
4	12.3	4	7.2	4	19.3
5	11.0			5	18.4
6	11.6	Control Average	8.3	6	15.6
Control Average	11.8	5 *	109.4	Control Average	18.1
7*	128.0	6	11.8	7 @	106.0
		7	11.5		
8	14.6	8	9.9	8	31.5
9	11.7	9	14.8	9	19.8
10	11.7	10	16.9	10	18.3
11	14.3	11	12.3	11	29.3
12	13.6	12	15.2	12	24.4
13	12.8			13	13.4
14	8.2	Post I.V. Average	13.2	14	16.4
15	10.4				
16	11.5			Post I.V. Average	23.3
Post. I.V. Average	12.1				

* Day of injection: 162 mgms. of testosterone intravenously
* Day of injection: 188 mgms. of testosterone intravenously
@ Day of injection: 164 mgms. of testosterone intravenously

testosterone to determine when the peak excretion of conjugated 17-ketosteroids occurred. From the data in Table X it is evident that the peak excretion occurred during the first and second hours after injection.

Effect of Liver Disease on the Rate of Excretion of Conjugated 17-ketosteroids after Intravenous Testosterone in Human Subjects

Data on the conjugated 17-ketosteroid excretion in cirrhotic subject JG (Table X) indicated that the rate of metabolism of testosterone to a conjugated 17-ketosteroid might be decreased in liver disease as reflected by a lower than normal 17-ketosteroid excretion in the first and second hours after injection. To test this, a series of ten normal subjects and twelve liver disease patients were injected with intravenous testosterone. Conjugated 17-ketosteroid analyses were made on urines collected during the first and second hours after injection. One case of chronic glomerulonephritis was similarly examined.

Inasmuch as patients with severe liver disease frequently have a reduced renal clearance, simultaneous endogenous creatinine clearances were run on most of these subjects to estimate the influence of the kidney on the urinary excretion of the conjugated 17-ketosteroids.(Table XI)

Normal human subjects were able to metabolize intravenous testosterone to conjugated 17-ketosteroids at a much more rapid rate than patients with liver disease. The average excretion for ten normal subjects was 16.4% of the administered testosterone excreted as conjugated 17-ketosteroids during the first hour after injection, as compared to an average of 4.6% for twelve cases with liver disease. During the

TABLE I: HOURLY URINARY EXCRETION OF CONJUGATED 17-KETOSTEROIDS AFTER INTRAVENOUS TESTOSTERONE

HRS. AFTER I.V.	NORMAL PATIENT CE		NORMAL PATIENT CH		CIRRHOTIC CG	
	17 ketos mgms/hr.	% testo excreted as 17 ketos	17 ketos mgms./hr.	% testo excreted as 17 ketos	17 ketos mgms/24 hrs.	% testo excreted as 17 ketos
1	29.6	17.7%	35.1	18.4%	6.4	3.6%
2	31.3	19.0	22.3	11.6	13.1	8.4
3	15.9	9.5	12.3	6.3	11.5	7.3
4	10.4	6.1	11.3	5.8	5.7	3.1
5	10.5	6.2	5.3	2.5	4.8	2.5
6	6.4	3.6	3.6	1.6	1.8	0.4
7			3.4	1.5	3.8	1.8
8			2.9	1.2	2.2	0.7
9			3.1	1.3		
10			1.2	0.4		

TABLE XI: A COMPARISON OF THE RATE OF EXCRETION OF CONJUGATED 17-KETOSTEROIDS BETWEEN NORMAL SUBJECTS AND PATIENTS WITH LIVER DISEASE

PATIENT NO.	DIAGNOSIS	% ADMINISTERED TESTOSTERONE EXCRETED AS 17-KETOSTEROIDS		CREATININE CLEARANCE IN cc of PLASMA
		1st hour	2nd hour	
CE	Normal	17.7%	19.0%	123 cc
CH	Normal	18.4	11.6	152
CN	Normal	15.4	16.5	108
CR	Normal	18.1	17.7	111
CV	Normal	12.2	10.1	117
DB	Normal	11.7	16.7	123
DC	Normal	13.2	10.5	130
DG	Normal	13.5	17.5	
DL	Normal	18.0	12.9	
DS	Normal	25.7	13.8	
	Normal averages	16.4%	14.6%	123 cc

TABLE XI (CONTINUED)

PATIENT NO.	DIAGNOSIS	% ADMINISTERED TESTOSTERONE EXCRETED AS 17-KETOSTEROIDS		CREATININE CLEARANCE IN cc of PLASMA
		1st hour	2nd hour	
CG	Cirrhosis	4.1 %	8.9%	
CL	Cirrhosis	8.1	10.4	165 cc
CM	Cirrhosis	0.2	3.5	65
CP	Cirrhosis	6.1	6.9	115
CQ	Cirrhosis	2.6	2.7	69
CS	Cirrhosis	6.9	--	92
CT	Cirrhosis	1.9	3.2	77
CU	Cirrhosis	5.2	5.5	66
DM	Hepatitis	11.3	8.1	
DO	Cirrhosis	4.4	10.8	
DR	Cirrhosis	4.3	6.8	

TABLE XI (CONTINUED)

PATIENT NO.	DIAGNOSIS	% ADMINISTERED TESTOSTERONE EXCRETED AS 17-KETOSTEROIDS		CREATININE CLEARANCE IN cc of PLASMA
		1st hour	2nd hour	
CW	?CA. of liver & nephritis	0.7%	0.9%	141 cc
	Liver Disease Averages	4.6%	5.6%	99 cc
CZ	Chronic glomerulonephritis	1.4	1.5	14

second hour the excretion was 14.6% in the normals and 5.6% in the liver cases.

The endogenous creatinine clearance averaged 123 cc. of plasma cleared per minute in seven normals, and 97 cc. per minute in seven liver cases. There was an average of 19.5% reduction in creatinine clearance, against an average reduction of 72% in the excretion of 17-ketosteroids in the liver cases. The maximum reduction in creatinine clearance in any single case of liver disease (CH) was 47.1%. The reduction in 17-ketosteroid excretion was 98.7%. In case CH the clearances of creatinine and 17-ketosteroids approached each other. The reduction in creatinine clearance was 46.8%; and in 17-ketosteroids, 68.3%. Even in this case the difference was large.

Three patients with liver disease had normal creatinine clearances but exhibited a marked reduction in 17-ketosteroid excretion.

Evidently reduced renal clearance does not account for the reduction in 17-ketosteroid excretion in liver disease after intravenous testosterone. This is assuming that the 17-ketosteroids and creatinine are handled by the kidney by similar mechanisms. In man creatinine is excreted principally by the glomeruli, but also to a lesser degree by the tubules.

In order to determine which kidney mechanism was involved in the excretion of conjugated 17-ketosteroids, the plasma clearances were calculated in four normal cases and three cases with liver disease in which plasma 17-ketosteroid values were available. These calculations were done with the average two hour urinary excretion values and the average plasma 17-ketosteroid value at sixty minutes for the four

normals and the three liver cases separately.

Using the average plasma concentration value at sixty minutes was valid inasmuch as a plot of the logarithm of the concentrations at serial times followed a straight line during this two hour period of excretion.

In this manner a renal plasma clearance of conjugated 17-ketosteroids in the four normal cases was calculated to be 126 cc. per minute. In the three liver cases this figure was 100 cc. per minute. There is close agreement between the creatinine clearances and the conjugated 17-ketosteroid clearances in both groups.

Inulin, which is cleared entirely by the glomerulus, has a plasma clearance of 125 cc. per minute. Although the number of cases in which the renal clearance values for conjugated 17-ketosteroids was studied is small, the probability is great that these steroids are excreted by glomerular filtration.

In respect to the effect of the kidneys on the excretion of conjugated 17-ketosteroids it should be noted that the one case of severe kidney disease studied had a reduced urinary excretion of 17-ketosteroids but an elevated plasma concentration that was about twice normal levels. In contrast, in liver cases both values were decreased below normal. Apparently severe kidney disease does not limit the formation and conjugation of 17-ketosteroids from testosterone, whereas liver disease does.

Isolation of Free Steroids from Urine

An attempt was made to isolate and identify the steroids in

the urine of human subjects receiving intravenous testosterone. Urines were collected for only two hours after injection to reduce the amount of interfering non-steroidal material and endogenous steroids in the urine. An average of 31% of the testosterone was excreted into the urine as conjugated 17-ketosteroids during this period.

Neutral ether extracts of these urines were prepared in the usual manner. The combined neutral extracts weighed 257 mgms. of which 158 mgms. were 17-ketosteroids, as measured by the Zimmermann reaction.

Girard Separation of the Ketones from the Non-ketones

The neutral extract, 257 mgms., was dissolved in 6.0 ml. of methanol. 0.44 ml. of glacial acetic acid and 300 mgms. of Girard's reagent T were added. The solution was refluxed for thirty minutes at boiling temperature. The mixture was then cooled and poured into a separatory funnel containing ice water. 95% of the amount of 2N NaOH needed to neutralize 0.44 cc. of glacial acetic acid, as determined by direct titration with phenolphthalein, was added. The aqueous solution was extracted three times with ice cold ether. The ether solutions were washed with water, 2N sodium carbonate, and water, dried, and evaporated. This fraction contained the non-ketones and weighed 97 mgms.

The aqueous solutions were combined and acidified with 10% concentrated HCl by volume. After standing at room temperature overnight, the solution was extracted three times with ether. The ether solutions were washed with water, 2N sodium carbonate, and water, dried, and evaporated. This fraction contained the ketones and weighed 165 mgms.

Ketonic Fractions

Chromatography I: The ketonic fraction (165 mgms.) was chromatographed on 5 grams of Merck aluminum oxide. 50 cc. of solvent were used in each eluent fraction. (Table XII)

No crystalline material was obtained from this first chromatography of the crude ketonic fraction.

Fraction 1 (3.5 mgms.) was an α-β unsaturated ketone as indicated by its absorption in the infra-red and ultra-violet spectra. It contained no hydroxyl group by infra-red analysis and gave a positive Zimmermann reaction for 17-ketosteroids. Absorption in the fingerprint region of the infra-red was similar to $^{3,5}_{\Delta}$androstadienol-17. No $^{3,5}_{\Delta}$androstadienone-17 was available as a standard for comparison. With the above evidence, this fraction was assumed to contain impure $^{3,5}_{\Delta}$androstadienone-17, although positive identification was not possible.

Fractions 2 and 3 (6.0 mgms.) were combined and sublimed in high vacuum at 145°. The sublimate weighed 4.4 mgms. and was rechromatographed (Chromatography V).

Fractions 4 and 5 were combined (10.7 mgms.) and acetylated with 0.5 cc. of acetic anhydride and 1 cc. of pyridine. After standing overnight at room temperature, the pyridine solution was removed in vacuo and the residue transferred to a separatory funnel with ether. The ether was washed several times with dilute HCl to remove all the pyridine, then with water, 2N sodium carbonate, and water, dried, and evaporated in vacuum. These acetates were then sublimed in high vacuum at 145°C. The sublimate weighed 11.7 mgms. and was subjected to further purification by alumina chromatography (Chromatography III).

TABLE XII

Chromatography I: Steroid Isolation Study on Urine after Intravenous Testosterone

Fr.	Solvent		Infra-red Absorption in	Ultra-violet Absorption	Mgms. Wt.	Appearance
1	Hexane-Benzene	9:1	5.74;5.76	235 mu	3.5	Oil
2	" "	7:3	5.72	Neg.	6.0	Oil
3	" "	1:1	5.73	"		
4	" "	1:3	5.73;5.95 2.72	"	10.7	Oil
5	Benzene		5.73;5.95 2.71	"		
6	Benzene-Ether	9:1	5.72;5.96 2.71	"	132.3	Red Oil
7	" "	4:1	5.73;2.71		3.8	Oil
8	" "	7:3	5.74;2.72			
9	" "	1:1	5.75;2.72	"		
10	Ether		5.75			
11	Ether-$CHCl_3$	1:1	5.75			

Fraction 6 (132.3 mgms.) was acetylated as above. The crude acetate was sublimed in high vacuum. The sublimate (134.5 mgms.) was chromatographed on aluminum oxide (Chromatography II).

Fractions 7-11 were combined and weighed 3.8 mgms. These fractions could not be crystallized. Infra-red analysis indicated that the compound was a 17-ketosteroid with a hydroxyl group. The absorption pattern in the fingerprint region of the infra-red was similar to etiocholanol-3α-one-17. Ultra-violet absorption indicated that there were no α-β unsaturated steroids in this fraction. A Zimmermann reaction was positive for 17-ketosteroids. Although positive identification could not be made, these 3.8 mgms. were thought to be impure etiocholanol-3α-one-17.

The total weight recovered from Chromatography I was 156.3 mgms. or 94.9% of the starting material.

Chromatography II: 134.5 mgms. of acetates from fraction 6 of Chromatography I were absorbed onto 5 grams of Merck aluminum oxide. 50 cc. of solvent were used in the eluate fractions.

The data for the chromatography are given in Table XIV. Eleven crystalline fractions (3-13) were obtained, weighing a total of 119 mgms. These crystals had melting points ranging from 97°C to 164°C. From later findings, these crystals would appear to be mixtures of etiocholanol-3α-one-17 acetate and androsterone acetate. This crystalline material (119 mgms.) was saponified. (See below)

The last four fractions yielded an oil which would not crystallize, combined weight 17.1 mgms. Infra-red analysis indicated that there was an α-β unsaturated ketone in this fraction. The tracing in the

TABLE XIV

Chromatography II: Urinary Steroid Isolations

Following Intravenous Testosterone in Human Subjects

Fr. No.	Solvent	Melting Point	Weight
1	Hexane	Traces	
2	Hexane	Traces	
3	Hexane-Benzene 9:1	97-108°	
4	" " 4:1	103-111°	
5	" " 7:3	108-120°	
6	" " 7:3	111-122°	
7	" " 7:3	108-121°	
8	" " 7:3	109-123°	119.0 mgm.
9	" " 3:2	112-122°	
10	" " 1:1	120-137°	
11	" " 1:1	122-142°	
12	" " 2:3	136-153°	
13	" " 3:7	153-164°	
14	" " 1:4	oil	
15	" " 1:9	"	17.1 mgm.
16	Benzene	"	
17	Ether	"	

fingerprint region of the infra-red spectrum was similar to, but not absolutely identical with, authentic testosterone acetate.

An absorption spectra in the ultra-violet range was run on this oil. A smooth curve was obtained with a maximum absorption at 240 mu., typical of α-β unsaturated ketonic steroids. Calculations from the ultraviolet absorption data indicate that fractions 14-17 contained 2.3 mgms. of α-β unsaturated steroid as free testosterone.

Quantitative Zimmermann determinations on this fraction indicated that there were 4.3 mgms. of 17-ketosteroids as free androsterone in these fractions. The identity of these 17-ketosteroids was unknown.

101% of the total starting material was recovered from the column.

Chromatography III: 11.7 mgms. of acetate from fractions 4 and 5 of Chromatography I were chromatographed on 350 mgms. of Merck aluminum oxide. 5 cc. eluate solvent fractions were used. This chromatography was carried out in the same manner as Chromatography II.

Nine crystalline fractions, melting in the range of 143-165°C and weighing 10.0 mgms. were obtained. This material was apparently androsterone acetate and was saponified (see below).

The fraction eluted with hexane-benzene 3:7 yielded a few crystals, m.p., 200-206°, weight 1.7 mgms. No definitive information about this material could be learned from an infra-red analysis except that it was an acetate. An ultra-violet analysis was negative for an α-β unsaturated steroid. The Zimmermann reaction was positive for 17-ketosteroids, identity unknown.

Saponification of Acetates: The crystalline fractions of

Chromatography II and III were combined (weight 129.0 mgms.) and saponified with 5 cc. of 5% KOH in ethanol at room temperature for two days. To neutralise the solution a few drops of water were added and gaseous CO_2 was bubbled through the solution. The neutral solution was then extracted three times with ether. The ether solutions were washed with water, dried , and evaporated to dryness. The saponified material weighed 112.0 mgms., 86.8% of the starting material.

Digitonin Precipitation: The above material (112 mgms.) was dissolved in 10 cc. of 90% ethanol and 75 mgms. of digitonin dissolved in 5 cc. of 90% ethanol were added. After standing at room temperature for two hours only a trace of precipitate had formed. 15 mgms. of dehydroisoandrosterone was treated the same way as a control and was quantitatively recovered as the precipitated digitonide in this period of time.

The precipitate was filtered off and washed with 90% alcohol. It weighed less than 0.5 mgm.

The filtrate was evaporated to dryness in vacuo. The residue was dissolved in 2 cc. of absolute pyridine and 20 cc. of absolute ether were added. The digitonin was removed by centrifugation and treated twice more with pyridine and ether in the same way. The combined ether solutions were washed several times with 2N HCl and water, dried, and evaporated to dryness. The residue contained the α-ketones and weighed 106 mgms.

Chromatography IV: The 106 mgms. of α-ketones were chromatographed on 5 grams of Merck aluminum oxide. The procedure of chromatography is given in Table XV.

TABLE XV

Chromatography IV: Isolation of Steroids from Human Urine after Intravenous Testosterone

Fr. No.	Solvent		Melting Point	Weight
1	Hexane-Benzene	9:1	Traces	
2	" "	7:3	"	
3	" "	1:1	"	
4	" "	2:3	"	
5	" "	3:7	"	
6	" "	1:4	"	
7	" "	1:9		
8	Benzene		178-185°	
9	"			
10	"			
11	"			19.8 mgm.
12	"			
13	"			
14	"		170-183°	
15	"		182-185°	
16	"	Ether 99:1	178-185°	
17	"	" 99:1	132-177°	5.5 mgm.
18	"	" 98:2	124-172°	
19	"	" 98:2	112-120°;141-148°;162°	
20	"	" 97:3	118-122°;141-143°	
21	"	" 97:3	116-119°	29.2 mgm.
22	"	" 97:3		
23	"	" 97:3	126-135°;141-142°	
24	"	" 97:3	139-149°	
25	"	" 96:4	138-140°;140-152°	
26	"	" 96:4		
27	"	" 95:5		45.8 mgm.
28	"	" 95:5		
29	"	" 9:1		
30	"	" 4:1	142.5°;151-152°	
31	Ether		138-155°	1.7 mgm

NOTE: Fractions 1 - 7 50 cc. Solvent
Fractions 8 - 31 25 cc. Solvent

Fractions 1-7 weighed less than 1.4 mgms. and could not be crystallised. They were discarded.

Fractions 8-16 weighed 19.8 mgms. and were crystallized from ether-pentane mixtures. These crystals melted from 178°C - 185°C and proved to be pure androsterone.

Fractions 17-19 weighed 5.5 mgms. Crystals were obtained from ether-pentane which melted over a wide range, 124 - 177°C. These fractions contained mixtures and were rechromatographed (Chromatography VIII).

Fractions 20-23 (29.2 mgms.) were also considered mixtures although obtained in a crystalline form and were rechromatographed (Chromatography VII).

Fractions 24-30 weighed 45.8 mgms. and were readily crystallised from pentane-ether. These crystals exhibited a double melting point with a transition point in the region of 138° and a final melting point near 148°. These fractions proved to be pure etiocholanol-3α-one-17.

The last fraction (1.7 mgs.) was apparently impure etiocholanol-3α-one-17 from its melting point.

The total amount recovered from this chromatography was 104 mgms. or 98.1%.

Chromatography VII: Fractions 20-23 from Chromatography IV (weight 29.2 mgms.) were combined and chromatographed on 1.5 grams of Merck alumina (Table XVI).

The first four fractions, weighing 0.7 mgms., were combined with fractions 17-19 of Chromatography IV and rechromatographed (Chromatography VIII).

Fractions 5-7 weighed 4.9 mgms. and crystallized from ether-

TABLE XVI

Chromatography VII: Isolation of Steroids from Human Urine after Intravenous Testosterone

Fr. No.	Solvent		Melting Point	Weight
1	Hexane		Traces	
2	Hexane-Benzene	1:1	"	
3	Benzene		122-130°	0.7 mgm.
4	Benzene-Ether	99:1	Traces	
5	" "	99:1	181.5-185°	
6	" "	99:1	184-187°	4.9 mgm.
7	" "	99:1	181-186°	
8	" "	99:1	117-125°; 154°	
9	" "	99:1	117-123°; 141°	
10	" "	99:1		9.2 mgm.
11	" "	99:1	120.5-126°; 139-141°	
12	" "	99:1	120.5-147°	
13	" "	99:1	130-136°;142-148°	
14	" "	99:1	133-136°;143-150°	
15	" "	99:1	133-138°;144-148°	
16	" "	99:1	138-140°;143-151°	
17	" "	99:1		
18	" "	99:1		
19	" "	98:2	150-152.5°	
20	" "	98:2		14.7 mgm.
21	" "	97:3		
22	" "	97:3	140-142°;149-152°	
23	" "	97:3		
24	" "	9:1		
25	" "	4:1	140-141°;146-153°	
26	Ether		134-139°;149-151°	0.9 mgm.
27	Ethanol		Traces	

pentane. The crystals melted from 181.5°-186°C and consisted of pure androsterone. They were combined with fractions 8-16 of Chromatography IV.

Fractions 8-13 (weight 9.2 mgms.) yielded crystals with a wide range of melting. These were mixtures and were rechromatographed with the Technicon fraction collector (Chromatography IX).

Fractions 14-25 weighed 14.7 mgms. and consisted of pure etiocholanol 3α-one-17. They were combined with fractions 24-30 of Chromatography IV.

Fraction 26 was apparently impure etiocholanol-3α-one-17, weight 0.9 mgms.

A total of 29.4 mgms. or 101% of the material was recovered from the column.

Chromatography VIII: Fractions 17-19 of Chromatography IV and fractions 1-4 of Chromatography VII (0.7 mgms.) were combined (weight 7.9 mgms.) and chromatographed on 400 mgms. of merck aluminum oxide. 5 cc. eluate fractions were used. (Table XVII)

Fractions 1-6 (weight 1.9 mgms.) were combined with fractions 8-13 of Chromatography VII and rechromatographed with the Technicon fraction collector (Chromatography IX).

Fractions 7-15 (weight 4.1 mgms.) consisted of pure androsterone and were combined with fractions 8-16 of Chromatography IV.

Fractions 16-25 (weight 3.0 mgms.) contained mixtures which were combined with fractions 8-13 of Chromatography VII and rechromatographed with the Technicon fraction collector (Chromatography IX).

Chromatography IX (Technicon): Fractions 8-13 of Chromatography VII, and fractions 1-6 and 16-25 of Chromatography VIII were combined

TABLE XVII

Chromatography VIII: Isolation of Steroids from Human Urine after Intravenous Testosterone

Fr. No.	Solvent		Melting Point	Weight
1	Hexane		Oil	
2	"		"	
3	Hexane-Benzene	3:2	Traces	1.9 mgm.
4	" "	1:4	Oil	
5	" "	1:9	Traces	
6	" "	1:9	"	
7	Benzene		173-184°	
8	"		174-185°	
9	"		184-186°	
10	"			
11	Benzene-ether	99:1	182-185.5°	4.1 mgm.
12	" "	99:1		
13	" "	99:1	180-185°	
14	" "	99:1	175-185.5°	
15	" "	99:1	174-185.5°	
16	" "	99:1	159-184°	
17	" "	99:1		
18	" "	99:1		
19	" "	98:2	115-163°	
20	" "	97:3	118-146°	3.0 mgm.
21	" "	95:5		
22	" "	9:1	132-151°	
23	" "	1:1		
24	Ether			
25	"			

(total weight 14.1 mgms.) and chromatographed on 8 grams of Baker and Adams aluminum. As an eluent a solvent mixture of 10% chloroform in hexane was used. 110 fractions were collected with the Technicon fraction collector. The first 65 fractions contained 2 cc. of solvent each. Fractions 66-91 contained 5 cc. each and fractions 92-110 contained 10 cc. each.

The first two fractions contained nothing and were discarded.

Fractions 1-26 (weight 1.6 mgms.) failed to crystalize. A substance absorbing maximally in ultraviolet region at 235 mu was present in these fractions. Infra-red analysis revealed that there was a 17-ketosteroid without a hydroxyl group. The absorption in the fingerprint region was quite comparable to $\Delta^{3,5}$androstadienol-17. No $\Delta^{3,5}$androstadienone-17 was available for comparison. The Zimmermann reaction was positive for 17-ketosteroids. It was felt that this was $\Delta^{3,5}$androstadienone-17, although it could not be positively identified.

Fractions 27-29 contained only traces and were discarded.

Fractions 30-64 crystallized from ether-pentane with melting points ranging from 178.5° to 186° (weight 7.2 mgms.). These crystals were pure androsterone and were combined with fractions 8-16 of Chromatography IV.

Fractions 65-69 (weight 1.0 mgms.) could not be crystallized. That a 17-ketosteroid was present was indicated by both a positive Zimmermannreaction and the absorption in the infra-red region. There was also a hydroxyl group present as detected by the infra-red analysis. The tracing of the absorption in the fingerprint region was similar to that of authentic etiocholanol-3α-one-17. This material was probably

etiocholanol-3α-one-17.

Fractions 70-104 (weight 7.4 mgms.) exhibited double melting points at 138°-142°C and 140°-153°C, typical of etiocholanol-3α-one-17. They were combined with fractions 24-30 of Chromatography IV.

Fractions 105-110 contained only traces of material and were discarded.

Chromatography V: Fractions 2 and 3 (weight 6.0 mgms.) from Chromatography I were sublimed at 145° in high vacuo. The sublimate weighed 4.4 mgms. and was chromatographed on 150 mgms. of Merck alumina. 5 cc. fractions of solvent containing increasing amounts of benzene in hexane were used to elute the material.

A single crystalline fraction was obtained by eluting with 7.1 hexane-benzene. It had a melting point of 70-84° and weighed 2.5 mgms. It gave a positive Zimmermann reaction for a 17-ketosteroid and the absorption in the infra-red region indicated a 17-ketosteroid lacking a hydroxyl. The absorption in the ultra-violet end of the spectrum indicated that there was an α-β unsaturated structure present.

From these data this fraction was considered to be $\Delta^{3,5}$androstadienone-17. Sufficient amounts were not present for more conventional methods of identification. (See Table XVIII for flow sheet of isolation procedure.)

Identification of Androsterone: The total amount of crystalline androsterone isolated from four chromatographies was 36.0 mgms.

This material was sublimed in high vacuo at 180°C and recrystallized from acetone-hexane. The crystals melted at 183°-185°C. An authentic sample of androsterone melted at 182.5°-185°C. The mixed

TABLE XVIII

Flow Sheet for Isolation Procedure for Urinary Steroids

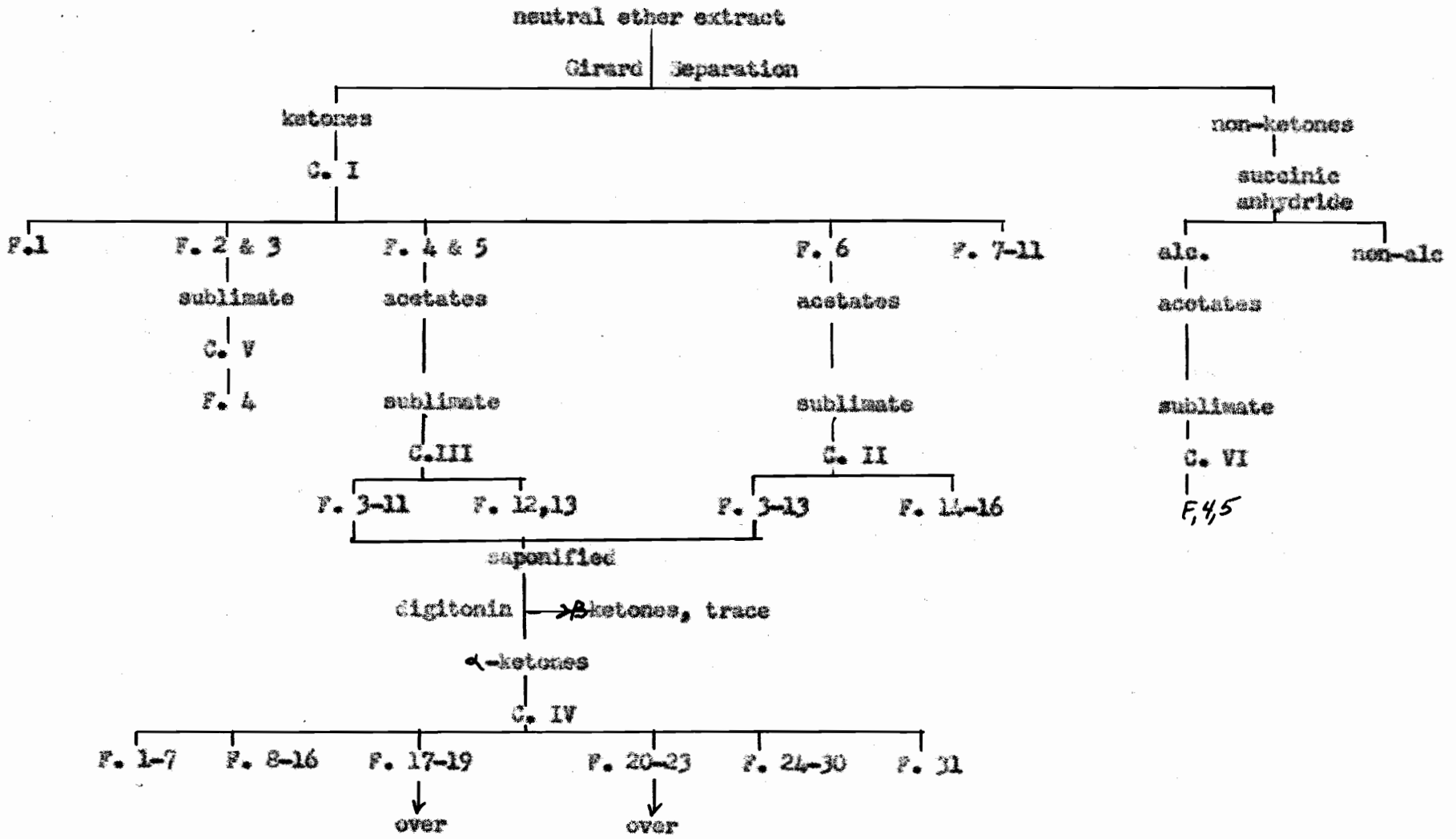
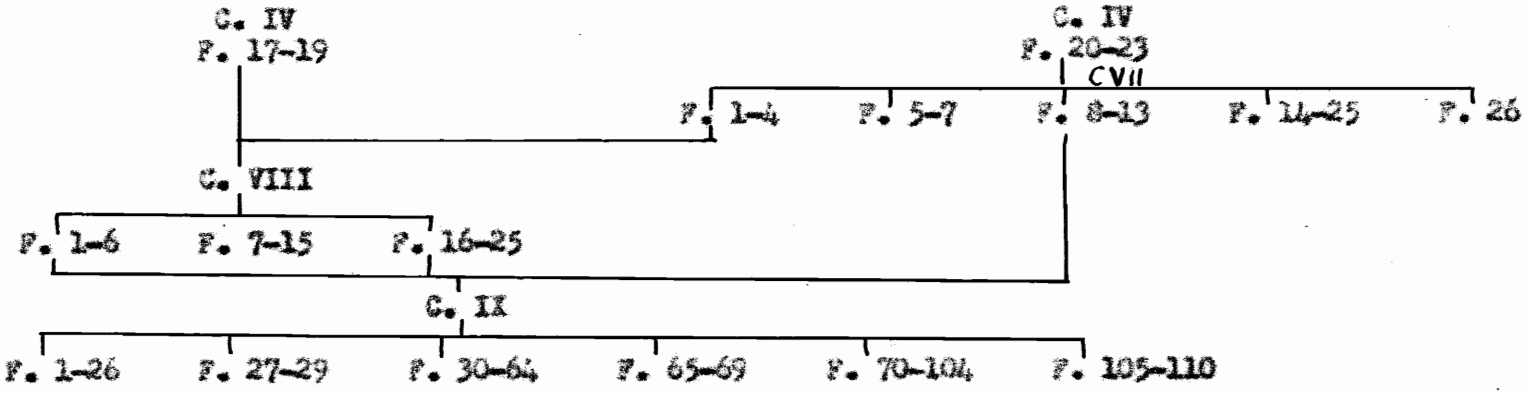


TABLE XVIII (Cont.)



NOTE: F = Fraction C = Chromatography

melting point was 182°-185°C, exhibiting no significant depression.

The acetate derivative was made in the usual manner. After purification by chromatography on Merck's alumina and recrystallization from dilute methanol, the acetate melted at 164°-166°C. An authentic sample of androsterone acetate melted at 163°-165°C. Upon mixing, there was no depression of the melting point (163°-166°).

Identification of Etiocholanol-3α-one-17: The total amount of crystalline etiocholanol-3α-one-17 obtained from three chromatographies was 70.5 mgms. This material was sublimed in high vacuo at 180°C and recrystallized from acetone-hexane. White needles exhibiting a double melting point were obtained (melting point 139°-143.5°C/149°-152°C). An authentic sample of etiocholanol-3α-one-17 melted at 139°-143.5°C and 147.5°-152°C. The mixed melting point was 138.5°-142.5°C and 147.5°-152.5°C.

The acetate derivative was prepared in the usual manner; and after chromatographic purification and recrystallization from dilute methanol, it melted at 93-95°C. An authentic sample of etiocholanol-3α-one-17 acetate melted at 94°-97°C. The mixed melting point was 93°-96°C.

The 3,5 dinitrophenylhydrazine derivative was also made as follows: 5 mgms. of etiocholanol-3α-one-17 were dissolved in 1.0 cc. of ethanol. 12 mgms. of dinitrophenylhydrazine were dissolved in 1.5 cc. of ethanol and 3 drops of concentrated HCl were added. 0.5 cc. of this dinitrophenylhydrazine solution were added to the steroid solution. The solution was boiled for two minutes. An excess of water was added, the alcohol removed in vacuo, and the dinitrophenylhydrazine derivative filtered off.

After purification by chromatography and recrystallization from diluted methanol, yellow crystals melting at 233°-235°C were obtained. Lieberman and Dobriner (74) reported the synthesis of this derivative in 1948 and gave its melting point as 225°-226°C.

The only other crystalline product obtained was 0.5 mgm. of material tentatively identified as $\Delta^{3,5}$androstadienone-17.

Table XIX summarized all the compounds here isolated from the ketonic fraction of the neutral steroid extracts of human urine following intravenous testosterone. The total weight of this ketonic fraction was 165 mgms.; 158 mgms. of this fraction were 17-ketosteroids as measured by the Zimmermann reaction, assuming that the Girard separation of the neutral steroid extract was complete. This figure of 158 mgms. can't be regarded as entirely accurate because of the indirect manner in which it was measured.

From the data in Table XIX it can be seen that 83.9% of the original ketonic fraction and 79.0% of the 17-ketosteroids were accounted for. The rest of the material was lost during purification.

78.9% of the total ketonic fraction was identified as 17-keto-steroids. 8.4% was non-17-ketosteroids. Of this figure, 1.4% was testosterone and 7.0% was unidentified material.

Of the 79% of the original 17-ketosteroids accounted for, 68.9% was isolated as crystals and 10.1% failed to crystallise and had to be identified by other methods.

Etiocholanol-3α-one-17 was the largest single component isolated, accounting for 47.7% of the original 17-ketosteroids in the ketonic fraction.

TABLE XIX

Steroids Isolated from the Neutral Steroid Extracts of Human Urine Following Intravenous Testosterone

Substances	Mgms. Crystalline Material	Mgms. non-Crystalline Material	Totals	% of Tot. Ket. Fr.
I. 17-Ketosteroids				
(a)etiocholanol-3α-one-17	70.5	4.8	75.3	45.6
(b)androsterone	36.0	0	36.0	21.8
(c) $\Delta^{3,5}$androstadienone-17	2.3	5.1	7.4	4.5
(d)unidentified 17-ketosteroids	0	6.0	6.0	3.6
Totals	108.8	15.9	124.7	75.5
% of total 17-ketosteroids in ketonic fraction	68.9%	10.1%	78.9%	
II. Non-17-ketosteroids				
(a)testosterone		2.3		1.4
(b)unidentified material		11.5		7.0
Totals		13.8		8.4
III. Alcoholic non-ketones				
(a)etiocholanediol-3α,17α	6.7		6.7	

Androsterone, the next largest component, accounted for 22.8% of the total original 17-ketosteroids.

Smaller amounts of $\Delta^{3,5}$ androstadienone-17 were found. 3.6% of the original 17-ketosteroids could be demonstrated by their absorption behavior in non-crystalline fractions but could not be identified.

Non-ketonic Fractions

The non-ketonic fraction from the Girard separation on the original 257 mgms. of neutral ether extract weighed 97 mgms. The alcoholic non-ketones were separated from the non-alcoholic non-ketones by formation of the hemisuccinates with succinic anhydride.

97 mgms. of non-ketones and 100 mgms. of succinic anhydride were dissolved 0.5cc. of pyridine and refluxed at 130°C for 3 hours. The material was dried in vacuum and transferred to a separatory funnel with ether. The ether solutions were washed several times with 2N HCl, H_2O, and four times with 2N sodium carbonate, collecting each alkaline wash. The alkaline washes were acidified to Congo red paper with dilute sulfuric acid and extracted three times with ether. The ether washes were washed with water, dried, and evaporated.

The ether extracts from the alkaline washes contained the alcoholic non-ketones (weight 53.9 mgms.) as hemisuccinates.

The original ether solution contained the non-alcoholic, non-ketonic material (weight 59.9 mgms.). This material was not investigated further and was discarded.

5 cc. of 5% KOH in methanol was added to the hemisuccinates (weight 53.9 mgms.) and hydrolysis was effected by heating on a boiling water bath under reflux for two hours. Following hydrolysis the

solution was neutralised with CO_2 gas and the free steroids extracted in the usual manner with ether. The ether extracts were washed, dried, and evaporated. The weight of the residue was 30.5 mgms. and contained the neutral alcoholic, non-ketonic steroids. The ether non-extractable material was discarded.

The 30.5 mgms. of alcoholic non-ketones were then acetylated in the usual manner. The acetates (33.5 mgms.) were sublimed in high vacuum up to 180°C. The sublimate weighed 25.0 mgms. That material which did not sublime was discarded.

Chromatography VI: The alcoholic, non-ketonic acetates (weight 25.0 mgms.) were next chromatographed on 1.0 gram of Merck aluminum oxide. The material was applied to the column dissolved in 25 cc. of hexane. Elution was carried out with successive 25 cc. fraction of solvents containing increasing amounts of benzene in hexane. Fourteen such fractions were collected. Two crystalline fractions were obtained with melting points ranging from 117°-126.5°C and weighing 7.5 mgms. On recrystallizing from dilute methanol white crystals in the form of needles were obtained, melting at 122.5°-126°C.

An authentic sample of etiocholanediol-3α,17α diacetate, kindly supplied by Dr. R. I. Dorfman, melted at 123.5°-126°C. The mixed melting point showed no depression (122.5-126°C). The alcoholic non-ketonic steroid isolated from urine was considered to be etiocholanediol-3α,17α by this identification of the diacetate. The amount was too small to permit further identification procedures.

The other chromatographic fractions were unidentified.

Thus, from 97 mgms. of non-ketonic material, 6.7 mgms. were

identified as etiocholanediol-3α,17α and the rest of the material was unidentified.

Isolation of Conjugated 17-ketosteroids from Urine Following Intravenous Testosterone

The urine from two normal patients receiving intravenous testosterone was examined for conjugated 17-ketosteroids. The urines in each case were collected for four hours after injection. The urine from each patient was worked up separately.

Conjugated 17-ketosteroids from Patient Number 1: In the first patient an aliquot of urine containing 50.1 mgm. of conjugated 17-ketosteroids, as determined by the Zimmermannreaction, was adjusted to a pH of 1.0 with concentrated hydrochloric acid. This acid urine was extracted five times with 250 cc. of n-butanol. The butanol extracts were combined and washed with 2.5% sodium hydroxide, 6% sulfuric acid, and water. The butanol was removed by vacuum distillation and the residue dried in vacuo. This residue weighed 863 mgms. and was positive for 17-ketosteroids by the Zimmermannreaction. This residue was then dissolved in hot absolute ethanol. The ethanol insoluble portion was discarded. The ethanol soluble fraction weighed 566 mgms. The ethanol was removed in vacuo and the residue was dissolved in 40% ethanol in acetone. This solution was chromatographed on 15.0 grams of Merck aluminum oxide. The eluent fractions consisted of 50 cc. each of solvent containing increasing amounts of ethanol in acetone, progressing from absolute acetone to absolute ethanol. The last fraction was 4% acetic acid in ethanol. The first two fractions eluted with acetone

alone contained all the 17-ketosteroids. The other fractions were discarded as they contained no 17-ketosteroids.

The first two fractions containing the 17-ketosteroids were combined and evaporated to dryness. The residue was dissolved in water. That material which was not water soluble was discarded. The water solution was extracted with ether three times and the ether extracts discarded.

The water soluble, ether insoluble fraction was evaporated to dryness in vacuo. The total weight was 78 mgms. with 9.5 mgms. of 17-ketosteroids (as androsterone) by the Zimmermann reaction. This fraction was combined with a similar fraction from the urine of patient number two before further purification was attempted.

Conjugated 17-ketosteroids from Urine of Patient Number 2: An aliquot of urine whose conjugated 17-ketosteroid content was 57.1 mgms. was adjusted to a pH 1.0 and extracted five times with n-butanol. The butanol extract was not washed but was brought to neutrality by adding 2N NaOH. The butanol was removed by vacuum distillation. The dried residue weighed 760 mgms. and gave a positive Zimmerman reaction.

This residue was dissolved in hot absolute ethanol. The ethanol insoluble portion was discarded. The ethanol soluble fraction weighed 502 mgms. and analyzed for 14.4 mgms. of 17-ketosteroids, as androsterone.

The ethanol was removed in vacuo and the residue dissolved in water. That material which was insoluble in water was discarded. The water solution was extracted three times with ether and the ether

washes discarded. 3.9 mgms. of 17-ketosteroids were present in the ether solutions.

The water soluble, ether insoluble fraction was dried and weighed 465 mgms. with 10.9 mgms. of 17-ketosteroids.

This material was dissolved in 25 cc. of water saturated butanol and chromatographed on a starch column 36 x 1.5 cm. The eluate fractions consisted of 50 cc. of water saturated butanol each. A total of 8 fractions were collected. The column run very slowly under 5 lbs. of pressure from a nitrogen tank. It took 72 hours to collect 8 fractions.

The first two fractions were negative for 17-ketosteroids. Fractions 3-5 were positive. The next two fractions were negative. A final fraction of absolute water was also negative for 17-ketosteroids.

Fractions 3-5 were combined and weighed 80.9 mgms. with 10.6 mgms. of 17-ketosteroids.

Fractions 3-5 were combined with the water soluble, ether insoluble fraction from patient one. The combined weight was 159 mgms. with 20.1 mgms. of 17-ketosteroids as free androsterone.

Aliquots of this fraction were chromatographed on filter paper, and R_f values determined. Authentic samples of all the known conjugated 17-ketosteroids were run simultaneously, namely, sodium androsterone sulfate, sodium dehydroisoandrosterone sulfate, and sodium estrone sulfate.

From the data (Table XX) it is apparent the conjugated 17-keto-steroid obtained from the urine following intravenous testosterone had a markedly different R_f value from any of the known conjugated 17-keto-

TABLE XX

Filter Paper Chromatography of Conjugated 17-ketosteroids

Conjugated 17-ketosteroid	Type of Column	R_f Values
1. Sodium androsterone sulfate	Ascending	0.75
	Descending	0.77
	Average	0.76
2. Sodium estrone sulfate	Ascending	0.69
	Descending	0.66
	Average	0.68
3. Sodium dehydroisoandrosterone sulfate	Ascending	0.64
	Descending	0.65
	Average	0.65
4. Sodium salt of unknown conjugated 17-ketosteroid	Ascending	0.53
	Descending	0.50
	Average	0.52

NOTE: Amounts chromatographed were 25-100 μgms.

steroids. The obvious conclusion from this data was that this was a new conjugated 17-ketosteroid which had not been isolated before.

An attempt to crystallize the conjugated 17-ketosteroid from acetone-H_2O and acetone-ethanol at this stage was unsuccessful.

The combined material was then chromatographed on 5.0 grams of alumina (weight 159 mgms.). 25 cc. eluent fractions were collected. The eluent solvents were mixtures of ethanol and acetone with progressively increasing amounts of ethanol, through absolute ethanol to acetic acid-ethanol mixtures (Table XXI).

Fractions 1-17 were negative for 17-ketosteroids and were discarded.

The conjugate had to be eluted from the column with acetic acid ethanol mixtures. In former chromatographies on alumina the conjugated 17-ketosteroid was eluted earlier with small amounts of ethanol in acetone. The reason why elution required acetic acid in ethanol this time was probably because the free acid form was applied to the column, whereas formerly the sodium salt was chromatographed.

A total of 181 mgms. of material were obtained from fractions 18-28. Inasmuch as more material was obtained from the column than was applied, it was apparent that part of the alumina must have dissolved in the acid solvents and passed into the eluent.

21.6 mgms. of 17-ketosteroids were in fractions 18-28 as determined by the Zimmermannreaction. The Tollen's resorcinol test for glucuronic acid was positive for several of these fractions containing 17-ketosteroids. It was suspected that the 17-ketosteroid was conjugated with glucuronic acid.

TABLE XXI

Chromatography III: The Isolation of the Conjugated 17-ketosteroids from Urine Following Intravenous Testosterone

Fr.	Solvent	Mgms. Wt.	17-ketos.*	Remarks
1	8% ethanol in acetone	1.1	0	pp't left
2	16% " " "	3.2	0	" "
3	20% " " "	12.2	0	" "
4	22% " " "	4.4	0	" "
5	22% " " "	1.9	0	" "
6	22% " " "	1.7	0	" "
7	24% " " "	Trace	0	" "
8	28% " " "	"	0	" "
9	32% " " "	"	0	pp't all dissolved
10	36% " " "	"	0	
11	40% " " "	"	0	
12	48% " " "	"	0	
13	56% " " "	"	0	
14	64% " " "	"	0	
15	72% " " "	"	0	
16	86% " " "	"	0	
17	Ethanol	"	0	
18	2% acetic acid in ethanol	4.5		
19	4% " " " "	16.0	0.29	Neg. resorcinol test
20	6% " " " "	19.3		
21	10% " " " "	19.2	1.75	
22	15% " " " "	30.1	5.15	
23	20% " " " "	27.8	5.30	Post. resorcinol test
24	20% " " " "	38.6	3.50	
25	20% " " " "			
26	30% " " " "	25.6	5.63	Post. resorcinol test
27	50% " " " "			
28	100% " " " "			
	Totals	181 mgms	21.6 mgms	

*by Zimmerman (in mgms. androsterone)

Fractions 18-20 were discarded.

Fractions 21-28 were combined with ethanol. To get rid of the alumina in these fractions the residue was dissolved in both absolute ethanol and butanol. That material which did not dissolve was discarded. The ethanol and butanol soluble residues were dissolved in H_2O made alkaline to litmus with dilute NaOH and extracted with ether three times. The ether was discarded.

The ether-insoluble, water soluble material weighed 105 mgms. with 21.3 mgms. of 17-ketosteroids as androsterone. The material gave a positive resorcinol test for glucuronic acid in dilutions of 1:40. If the 17-ketosteroids were calculated as sodium androsterone glucuronidate instead of free androsterone, the material contained 35.9 mgms. of sodium androsterone glucuronidate and was 34.2% pure.

The material was dissolved in methanol and pH adjusted to 9.0 with dilute NaOH. The methanol solution was concentrated to approximately 0.5 cc. and allowed to stand overnight at 0°C. A flocculent crystalline material precipitated from solution and was removed by centrifugation. The crystalline material was washed three times with ice cold methanol and dried in a vacuum dissicator for 24 hours.

The dried crystals were a light tan in color and weighed 33.4 mgms. with 2.1 mgms. of 17-ketosteroids as androsterone by the Zimmermann reaction. There was considerable undissolved material in the reaction tubes.

An attempt was made to separate the conjugated 17-ketosteroids from the rest of the material by filter paper chromatography. The entire sample (33.4 mgms.) was applied as a band to one side of a square

piece of Whatman No. 2 filter paper. The chromatogram was developed with water saturated n-butanol as an ascending column.

From a vertical sample strip the position of the horizontal band of conjugated 17-ketosteroid was determined. This horizontal band was cut out and the material soaked off with absolute alcohol.

No separation of the conjugated 17-ketosteroid was achieved. The material obtained in this manner weighed 26.6 mgms. with 1.89 mgms. of 17-ketosteroids as androsterone. On heating for a melting point the crystals underwent a brownish decomposition. No melting was observed up to 350°C. The conjugated 17-ketosteroid content calculated as sodium androsterone glucuronidate was 3.2 mgms. By Naughn's quantitative analysis there were 1.88 mgms. of glucuronic acid in this crystalline fraction, equivalent to 4.72 mgms. of sodium androsterone glucuronidate. The average purity of this fraction as sodium androsterone glucuronidate on the basis of the Zimmermannreaction was 11.1% and on Naughn's test was 16.5% for an average of 13.8%.

The mother liquors and methanol washes from the first crystallization were combined and the same crystallization procedure repeated. The crystals from the second crop were less pigmented but still faintly tinged with brown. These crystals also failed to melt up to 350°C and underwent a brownish decomposition on heating.

The second crop of crystals weighed 41.9 mgms. and contained 23.3 mgms. of 17-ketosteroid as androsterone by the Zimmermannreaction, equivalent to 39.3 mgms. of sodium androsterone glucuronidate. By the Naughn test this material gave 15.6 mgms. of glucuronic acid, equivalent to 39.0 mgms. of sodium androsterone glucuronidate. The average purity

by these two quantitative colorimetric tests was 95.6% as sodium androsterone glucuronidate.

In a similar manner a third crop of crystals was obtained from the mother liquors and washes of the second crystallization. This crop was composed of faintly pigmented white crystals that failed to melt up to 350°C and underwent brown decomposition on heating.

The third crop of crystals weighed 12.0 mgms. of which 93.6% was sodium androsterone glucuronidate by the Zimmermann reaction and 86.6% by the Maughn test, for an average purity of 90.1% by these two colorimetric procedures.

Material from the second crystallization was hydrolyzed with β-glucuronidase and the free 17-ketosteroids isolated and identified. The β-glucuronidase was obtained from the Viobin Company and assayed 25,000 Fishman units per gram.

29.7 mgms. of crystals from the second crystallization were dissolved in 18.0 cc. of distilled H_2O. 12.0 cc. of acetate buffer (pH 5.0) and 0.750 grams of the glucuronidase preparation were added. The mixture was incubated at 37°C for 22 hours. The incubation mixture was then transferred to a separatory funnel and extracted three times with ether. The ether extracts were washed with water, dried, and evaporated. The dried ether extract weighed 10.4 mgms. and was chromatographed on 400 mgms. of Merck aluminum oxide. 5 cc. eluent fractions were collected (Table XXII).

Fractions 1-4 weighed 1.4 mgms. and failed to crystallize. No identification was made of these fractions.

Fractions 5-9 crystallized from pentane-ether, yielding crystals

TABLE XXII

Chromatography of Hydrolysed

Conjugated 17-ketosteroid

Fr. No.	Solvent		Melting Point	mgms. Weight
1	Hexane			1.4
2	Hexane-Benzene	7:3		
3	" "	1:1		
4	" "	3:7		
5	" "	1:9		
6	Benzene		182.5-183.5°C	3.0
7	"		175-183.5°C	
8	"			
9	Benzene-Ether	99:1	172-183°C	
10	" "	99:1	114-179°C	1.0
11	" "	98:2	118-159°C	
12	" "	97:3	114-116-133°C	4.0
13	" "	95:5	121-142°C	
14	" "	95:5	135-138°C 145-148.5°C	
15	" "	95:5		
16	" "	9:1	129-138°C 143-145°C	
17	" "	9:1		
18	" "	1:1		0.9
19	Ether		168-192°C	

melting from 172-183.5°C. These fractions were combined and weighed 3.0 mgms. Upon crystallization from acetone-hexane the combined fractions melted at 174-183°C. These crystals were recrystallized from ether-pentane and melted at 181-183°C. An authentic sample of androsterone melted at 182°-184°C. When the two were mixed there was no significant depression (mixed m.p. 181-184°C). Fractions 5-9 were identified in this manner as androsterone.

Fractions 10-11 (wt. 1.0 mgms. melted in a range from 114-179°C. and were considered to be mixtures of androsterone and etiocholanol-3α-one-17.

Fractions 12-17 exhibited a double melting point, with a transition point at 129-138°C. and a final melting point at 143.5-148.5°C. This double melting point is one characteristic of etiocholanol-3α-one-17. Fractions 12-17 were combined and crystallized from acetone-hexane with a melting point of 133-138°C., tr. pt./143-148°C. These crystals were recrystallized from pentane-ether and melted at 135-139°C., tr. pt./145-147°C. An authentic sample of etiocholanol-3α-one-17 melted at 137-139°C., tr. pt./147-151°C. The mixed melting point was 133-138°C., tr. pt./143-147°C. In this manner fractions 12-17 were identified as etiocholanol-3α-one-17.

Fractions 18-19 yielded crystals melting at 168-192°C. and weighing 0.9 mgms. Further identification was not made.

The identification of androsterone and etiocholanol-3α-one-17 after the enzymatic hydrolysis of the conjugated 17-ketosteroid with β-glucuronidase established that a mixture of androsterone and etiocholanolone glucuronidates had been formed.

The importance of conjugation with glucuronic acid in the metabolism of intravenous testosterone is indicated by the fact that 31.0% of the original urinary 17-ketosteroids were isolated in the form of glucuronidates.

DISCUSSION

This [redacted] 17-ketosteroids are the principal metabolites of testosterone.

In any study of the metabolism of testosterone in the human in which steroid metabolites are isolated from the urine following testosterone administration, steroids arising from endogenous sources must be considered. In the past, endogenous steroids were a serious interference in determining whether a given isolated steroid resulted from metabolism of the administered testosterone or from endogenous sources. Testosterone had to be administered by some other route than the intravenous one and was slowly absorbed into the body, utilized, and excreted. To be sure of obtaining all of the product from the administered hormone, Urine collections had to be made for several days, both during and after treatment. The longer the time period for collection the greater was the excretion of endogenous steroids.

Consider the influence of interfering endogenous steroids from actual cases in the literature. Dorfman (99) has summarized the data from six experiments in the literature (89-90-91-92).

In these experiments an average of 400 mgms. of testosterone was given in each experiment as testosterone propionate, either orally or intramuscularly. Of this, an average of 15.6% of the administered testosterone was recovered as steroid metabolites from the urine. The urines were collected for an average of nine days.

Four of the cases were hypogonadal male hypogonads, one was a male castrate, and the third was a woman. No normal 17-ketosteroid excretion values

during a control period were reported for any of these subjects. Certain approximations can be made from the literature. Nine normal women excreted an average of 9.6 mgms. of 17-ketosteroids per 24 hours; fourteen eunuchs excreted an average of 6.5 mgms. per 24 hours; and ten male castrates, an average of 9.1 mgms. per 24 hours (99). On the basis of these figures an average of 7.5 mgms. per 24 hours would be expected to arise from endogenous sources in these six subjects.

Over the nine day period of urine collection an average total of 67.5 mgms. would be expected to originate from endogenous sources. The average total amount of steroids isolated in these experiments was only 62.4 mgms.

Even in the best experiment the greatest single steroid component isolated amounted to 146 mgms. In this experiment there would be 86.4 mgms. of endogenous steroids in the urine. In this experiment it is quite safe to assume that at least part of the steroid originated from testosterone metabolism. However, other steroids such as isoandrosterone, etiocholanediol-3α,17α, and androstenediol-3α,17α, have been isolated in much smaller amounts following treatment with testosterone.

More recently Dobriner and Lieberman have reported that androsterone and etiocholanol 3α-one-17 are the major ketonic metabolites excreted after the intramuscular administration of testosterone (155). The excretions of androstanedione, etiocholanedione, isoandrosterone, etiocholanol-3β-one-17, and dehydroisoandrosterone were not altered during the injection period. 44% of the testosterone injected could be accounted for as ketonic metabolites, 23.7% as androsterone and 19.3% as etiocholanol-3α-one-17. The two non-ketonic alcohols, androstanediol

and etiocholanediol, which were also found, accounted for only 5.5% of the testosterone. Only 50% of the injected testosterone could be accounted for, however, either as ketonic or as alcoholic non-ketonic metabolites.

This contrasts strongly with the results after intravenous injection of the hormone where, in every case, close to 75% was recovered as excess 17-ketosteroids alone. Two explanations for the discrepancy between the two types of administration are possible. First, the excretion was not increased as sharply in the intramuscular injections because of the slow absorption, and variations in endogenous output would obscure a larger proportion of the increase. In the presence of such large amounts of exogenous hormone being absorbed at a fairly steady rate, the endogenous production may have been depressed to negligible levels; yet the calculation of recoveries assumes a basic level equal to the pre-injection period. Thus the amount arising from the injected hormone would have been greater than the calculated amount.

A second factor may be that at the blood levels which must have existed in the subject receiving intramuscular injections the relative activity of a second system not producing 17-ketosteroids was greater than at the high initial levels achieved in the intravenous experiments. That such systems exist is indicated by the intravenous studies in cirrhotic patients and <u>in vitro</u> studies with liver and kidney tissue.

These experiments emphasize how difficult it is to determine whether a steroid isolated from urine following the administration of testosterone orally or intramuscularly originated from the administered

testosterone or from endogenous sources. Because of the large amounts of interfering endogenous steroids it is even more difficult, if not impossible, to determine the relative importance of an isolated steroid as a metabolic product of testosterone by trying to account for the percent of testosterone metabolized.

Another disadvantage to experiments of this type is that large volumes of urines containing high concentrations of non-steroidal contaminants must be handled. Because of these contaminants, not only would the purification procedure be more difficult, but also losses of steroidal material during purification would be great.

Because of the rapid rate at which intravenous testosterone is metabolized, it is possible to analyze urine specimens collected for only two hours after injection for their steroid content. During this time an average in normal subjects of 31% of the administered testosterone was excreted as 17-ketosteroids. The average dose of testosterone administered in this present study was 167 mgms., so that an average of 52 mgms. of 17-ketosteroids were excreted during the first two hours after administration.

During this two hour period the expected concentration from endogenous 17-ketosteroids would be almost negligible. In any two hours a normal male would be expected to excrete 1.2 mgms. of 17-ketosteroids from endogenous sources. This would amount to only 2.3% of the total 17-ketosteroids in the two hour urine.

In this present study a total of only 3.3 mgms. of the total 125 mgms. of 17-ketosteroids isolated and identified could have originated from endogenous sources. Even the smallest single component

isolated could not have arisen entirely from endogenous sources.

Thus, it can be stated with confidence that the 17-ketosteroids isolated in this study resulted from the metabolism of the administered testosterone.

Not only are the interfering endogenous steroids eliminated, but also the technical processing of the urine is made easier by the handling of smaller volumes with lower concentrations of contaminants. It would be expected that the recovery of steroids would be more complete.

In this investigation 79% of the 17-ketosteroids in the urine were actually isolated and identified. Assuming that the ~~steroid excretion~~ distribution between the first and second was the same in the entire post-injection period as it was in the first two hours after injection, 59.5% of the total administered testosterone was ~~identified~~ recovered as etiocholanol-3α-one-17, androsterone, and $\Delta^{3,5}$androstadienone-17.

The major 17-ketosteroid metabolite isolated was etiocholanol-3α-one-17. About half as much androsterone was isolated. No β-ketosteroids were found.

$\Delta^{3,5}$androstadienone-17 which was isolated by us after injection of testosterone has been found in the urines of most men. The precursor of this compound has been believed to be dehydroisoandrosterone sulfate. The amount recovered, however, considerably exceeds the total amount of dehydroisoandrosterone and $\Delta^{3,5}$androstadienone-17 which would have been normally excreted during the entire time represented by the combined collections. Since we were not able to isolate any considerable amount of dehydroisoandrosterone, it is assumed that

some testosterone might have been converted to Δ^4androstenol-3-one-17. It is known that this compound undergoes ready dehydration on acid hydrolysis to give $\Delta^{3,5}$androstadienone-17.

In the past approximately equal amounts of etiocholanol-3-one-17 and androsterone have been isolated from the urine (49-90-91) following testosterone therapy.

In the one study that reports the isolation of isoandrosterone from a castrated male following testosterone administration the isoandrosterone could have originated from endogenous sources. Callow (37) has shown that castrated males differ from normal men in that the β-ketosteroid content of the urine may increase to as high as 65% of the total 17-ketosteroids.

No other β-ketosteroid has been isolated from urine following testosterone administration.

It is interesting to examine the evidence for the various possible pathways in the metabolism of testosterone in an attempt to determine which pathways are actually involved in the metabolism of testosterone. In Figure 5 all the possible pathways for the metabolism of testosterone to 17-ketosteroids are illustrated and the evidence for the given reactions indicated.

The first chemical reaction which the testosterone molecule could undergo in the body would be either an oxidation of the 17-OH to a ketone or a reduction in ring A.

If oxidation were the primary reaction the first product in the intermediate metabolism of testosterone would be Δ^4androstenedione-3,17. There is good evidence to indicate that Δ^4androstenedione-3,17 is an

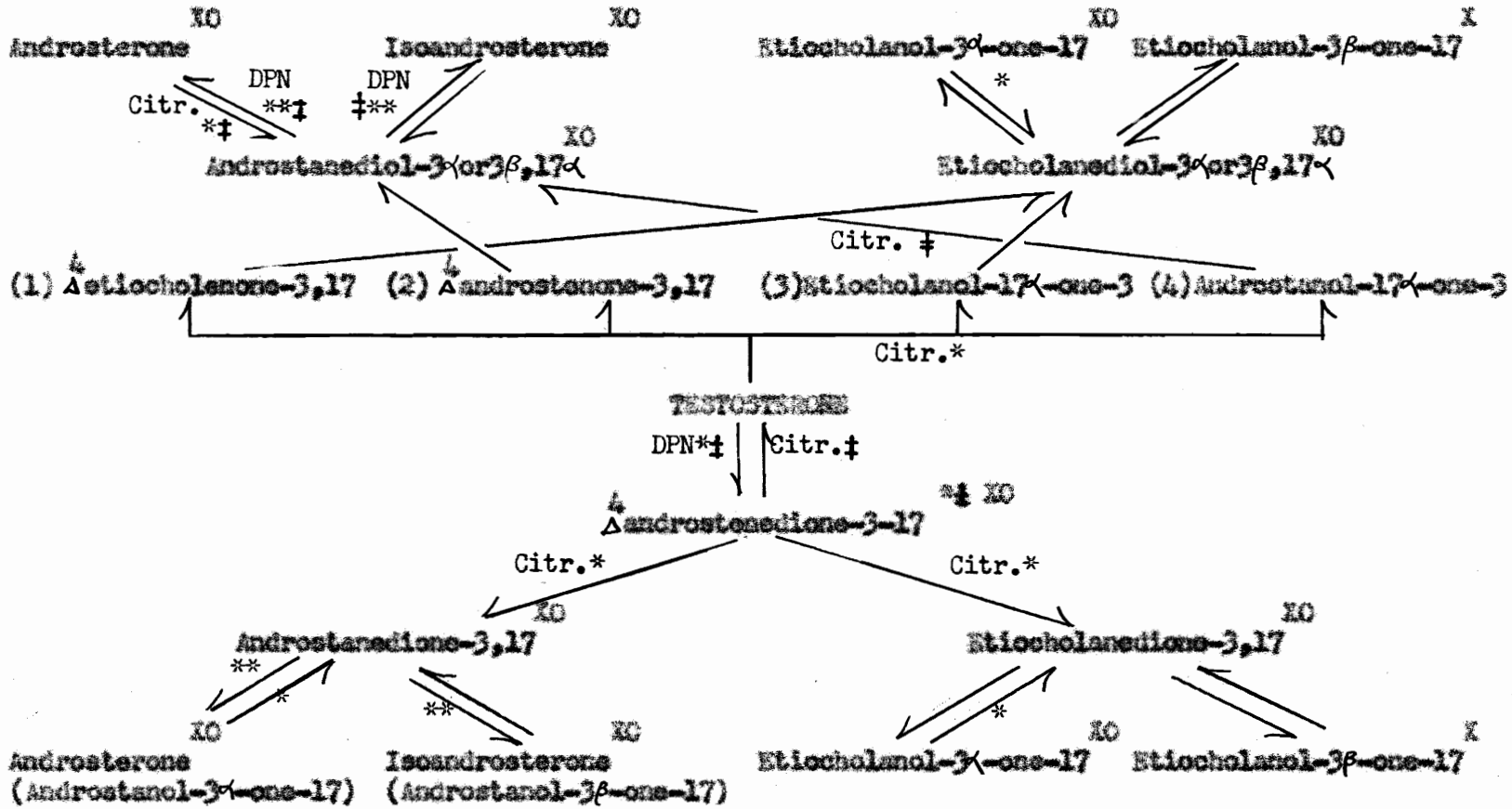


Figure 5: Possible Pathways in the Metabolism of Testosterone

* demonstrated in liver tissue incubations with testosterone
‡ " " kidney " " " "
X isolated from normal urine
O isolated from urine after testosterone administration
** reaction observed in vivo

important intermediate in the metabolism of testosterone. It has been isolated from both normal male urine and after the administration of testosterone (73-155). The administration of Δ^4androstenedione-3,17 results in the isolation of etiocholanol-3α-one-17 and androsterone in approximately the same proportions as following testosterone administration (100-101).

Δ^4androstenedione-3,17 has been isolated from incubations of both liver and kidney tissue with testosterone (108-116).

This evidence indicates that Δ^4androstenedione-3,17 is an important intermediate metabolite in testosterone metabolism. The question arises as to whether the major portion of the testosterone is metabolized by way of Δ^4androstenedione-3,17.

There is less evidence to support the alternate reduction of the A ring in testosterone as a major route of metabolism. Although the diols have been isolated both from normal urine and following testosterone therapy (73-92-116-156), the first reduction products of testosterone, have never been isolated from normal urines, urines after testosterone therapy, or from incubations. This does not necessarily mean that the major route is not by reduction. If the successive reactions to the diols and 17-ketosteroids were rapid enough, one would not expect to isolate the first products of reduction.

The fact that the saturated diols have been isolated in significant amounts following testosterone administration, both in this study and others, indicates that they play an important role in the metabolism of testosterone. The possibility exists that the diols are

not intermediates in testosterone metabolism but are reaction products of androsterone and its isomers.

Samuels and his coworkers (114-115-116), in their investigation of the enzyme systems involved in the metabolism of testosterone, have shown that the relative concentration cofactors, as well as enzyme concentration, is an important factor in determining the pathway taken in the metabolism of testosterone. It was found that DPN catalyzed the oxidation of testosterone to Δ^4androstenedione-3,17 by both liver and kidney tissue. On the other hand, citrate catalyzed the reduction in ring A in testosterone by the liver but not by the kidney.

Because the addition of both citrate and DPN resulted in the most rapid reduction in ring A, it was concluded that the α-β structure in Δ^4androstenedione-3,17, which was formed by the DPN system, was more rapidly reduced than that of testosterone. Under normal physiological conditions in which an excess of both factors would probably be present in respect to the steroid substrate the preferred route of metabolism would probably be via Δ^4androstenedione-3,17, all other factors being equal.

However, should the citrate concentration in the liver increase or the DPN concentration decrease to critical levels, it would be expected that more testosterone would be metabolized by the reduction route via the diols. Certainly enzyme systems to accomplish both types of reactions are available in the liver, and the metabolic route taken would depend upon the relative cofactor concentrations as well as other factors such as relative enzyme concentrations.

In a consideration of the metabolism of testosterone by the

entire body one cannot draw too many conclusions on the relative importance of various metabolic pathways from studies on any single tissue unless that tissue is the only site of metabolism. If more than one tissue is involved, the influence of all active tissues on the metabolism of testosterone must be considered insofar as the body as a whole is concerned.

Testosterone is metabolized by at least two different organs, the liver and the kidney. A study of a wide variety of tissues in incubation studies has failed to demonstrate metabolic activity in any others tissues. This does not rule out the fact that they may be active under proper conditions which have not been discovered.

A consideration of the known active tissues, liver and kidney, reveals interesting relationships in the metabolism of testosterone. Both tissues oxidize the 17-OH group under the influence of DPN. Liver has a citrate activated system which reduces the A ring. This citrate activated system is absent in kidney tissue. Citrate has a different action in kidney tissue. It catalyzes the reduction of 17-ketones, presumably reversing the DPN activated system.

It is interesting to speculate on what might occur in testosterone metabolism in the whole body if it were assumed that the liver and kidney were the only active tissues. Under the influence of DPN both tissues would form Δ^4androstenedione-3,17 from testosterone and the oxidative route would be the major pathway of metabolism insofar as the whole body was concerned.

Under the influence of citrate the kidney would force the reversible reaction from testosterone to Δ^4androstenedione-3,17 to

testosterone synthesis. The testosterone would presumably find its way to the liver by way of the circulation where it would be metabolized as discussed above.

If more tissues than these two were active in testosterone metabolism, the over all metabolism in the body would be very complex indeed.

From Figure 5 it can be seen that other evidence of an indirect nature is available to support both major metabolic pathways. The administration of both androstanedione-3,17 and androstanediol resulted in the isolation of androsterone (100-101). Incubations with androsterone and etiocholanol-3α-one-17 have resulted in the isolation of both respective diones as well as diols (119). Enzyme systems that oxidize the 17-OH group to a ketone in androstanediol-3α,17α and androstanol-17α-one-3 under citrate catalysis have been demonstrated in kidney tissue (115). The α-β unsaturated ring A of Δ^4androstanedione-3,17 is reduced by liver tissue (114).

Our data in this study indicated that the major route of testosterone metabolism to 17-ketosteroids was through the etiocholane nucleus. About half as much was metabolized by way of the androstane nucleus. In both cases the 3αisomer was formed. The fact that no 3βketosteroids were isolated indicates that their formation is of little importance in the metabolism of testosterone.

Our data do not demonstrate whether the testosterone molecule was oxidized or reduced to form 17-ketosteroids. The isolation of significant amounts of etiocholanediol-3α,17α indicates that at least a part of the testosterone could have been metabolized by the reduction

route.

It has been thought that the reduction of the keto group on C-3, like the double bond reduction, was not strictly directed. Dorfman (93) has reported the isolation of isoandrosterone from the urine of a hypogonadal man after the administration of testosterone propionate intramuscularly. Dobriner and Lieberman (155) have also isolated isoandrosterone and etiocholane-3β-ol-17-one from the urine of a male subject injected with testosterone propionate. In view of the fact that no more 3β-hydroxy ketosteroids were excreted during the period of injection than during the control period, however, it could not be definitely stated that these compounds had arisen from the metabolism of the administered testosterone. The in vitro studies with liver indicate that Δ^4-androstene-3,17-dione is an intermediate in the formation of the saturated 17-ketosteroids. It seems highly likely, therefore, that the reduction of the double bond leads to both isomers, but that the enzymic conversion of the ketone on C-3 to an alcohol is limited to the α configuration. The effects on the 3β forms cited above were probably the result of indirect influence on endogenous metabolism.

The only conjugates of androgens which have been isolated thus far are sodium androsterone sulfate and sodium dehydroisoandrosterone sulfate. It has been assumed, however, that the body utilizes more than one mechanism for making androgens water-soluble so that they can be excreted. Our findings confirm this assumption, as we could show that androsterone and etiocholanolone are also excreted in the form of their glucuronides. Unfortunately it was not possible to separate

these conjugates, although the mixture of both was 90% pure. This mixture obtained by direct crystallisation contained androsterone and etiocholanolone in the ratio of 3:4 as isolated after fission with β-glucuronidase.

Certain interesting physiological findings arose from this study on the metabolism of intravenous testosterone.

From the animal work the sequence of events in the fate of intravenous testosterone seems first to involve rapid diffusion from the circulation into the tissues, particularly the fat stores. This occurs during the first few minutes.

The mechanism of this early removal would appear to be two-fold. The great concentration in fat was apparently a function of the relative solubilities of the hormone in it and in plasma. As the concentration in the latter fell, the hormone readily moved back into circulation. Thus fat would serve as a buffer which would tend to level out variations in the blood level. The second would appear to be a protein binding similar to that with albumin, since the concentration in tissues other than the fat stores bore no relation to their relative fat content. This is probably associated with the metabolic action of the hormones. Such binding may be more lasting since action on nitrogen metabolism continues for several days after a single intravenous injection (158) long after levels in the blood and fat are insignificant.

The fact that all the testosterone which was found in the tissues after injection was readily extracted with ether before hydrolysis indicates that the chemical bonding between the tissues and the testosterone which was recovered was weak. Living tissue, however,

may also combine firmly with testosterone in such a way that it is destroyed during the hydrolytic process. Even in the nephrectomized, hepatectomized animal, less than half the injected hormone was recovered in tissues representing a large part of the animal's mass. Such a possibility can be ruled out only by use of a variety of hydrolytic procedures.

The data showing the decreased rate of disappearance of testosterone from the circulation of hepatectomized animals indicates that the liver is a site of metabolism in the whole animal. Nephrectomy had an additional effect in the hepatectomized animal. It would seem, therefore, that *in vivo*, as *in vitro*, both organs play a part in the metabolic action on the testosterone molecule.

One of the most striking findings in this investigation was the rapid rate of disappearance of the intravenous testosterone. Within five minutes after injection less than 10% of the theoretical blood level of testosterone was found. Referring to the animal experiments this early loss of testosterone from the circulation could be explained by diffusion of the testosterone into the tissues.

Once equilibrium was established, the rate of fall of the testosterone concentration in the blood was logarithmic, a result one would expect if this concentration were the limiting factor in the rate of the enzymic reactions. The work of Sweat *et al* (116) has shown that there are two major enzymes in the liver which act on the testosterone molecule; one requiring citrate as a cofactor which acts on the conjugated system in ring A without affecting the alcohol group on C-17, and another requiring DPN for its action which first oxidizes the alcohol

to a 17-ketone and then, by further enzymic reactions, reduces ring A to a saturated alcohol. From in vitro experiments it was concluded that the latter route, producing androsterone and its isomers, was the preferred route in the human body.

Since, in the normal individual, the concentrations of both testosterone and 17-ketosteroids fall at a logarithmic rate over the measurable range, it would appear that none of the intervening enzyme systems is saturated. Since the concentration of 17-ketosteroids is the algebraic result of their formation and their removal by the kidney, it must be true that the latter process is also proportionate to the concentration, a condition borne out by the renal clearances which indicate that these substances are filtered without being reabsorbed. Assuming a constant blood flow, the result observed would be anticipated. Further, one can conclude that the reaction involving conjugation must have its equilibrium far on the side of the conjugated product since the conversion of testosterone to these compounds was still only dependent on the testosterone concentration when the concentration of the conjugates was almost 20 times that of the hormone (Fig. 2).

The rate of disappearance of testosterone cannot, however, be taken as a measure of the rate of the limiting reaction in the formation of 17-ketosteroids, because the data also show that the hormone is disappearing by some other route. This is probably the citrate system which was found in the in vitro studies with liver minces. It seems to occupy a minor role in the normal human being, however, under these circumstances since most of the injected hormone could be accounted for in the conjugated 17-ketosteroids.

The role of the liver in these enzymic mechanisms is emphasized by the results in the cirrhotic patients. During the first sixty minutes the concentration of testosterone decreased along the same logarithmic line followed in the normal subjects. The concentration of 17-ketosteroids in the blood, however, followed an altogether different course from that in the normal individual. Instead of a logarithmic decrease from a high level, it was about one-fourth the normal level twenty minutes after injection and rose at an increasing rate thereafter for the first hour. The slow production was reflected in a very low excretion during the first hour, increasing during the second hour, and approximating the decreasing normal rate from the third hour on.

This type of curve could only be obtained with a falling testosterone level if some intermediate were accumulating, the breakdown of which is normally so rapid that it is not a limiting factor in the over-all reaction. As the concentration of this rises the conversion to 17-ketosteroids increases by mass action until the rate equals that of the normal without accumulation of the intermediate. Thus damage to the liver markedly decreases the activity of one or more of the enzymes involved in the conversion of testosterone to 17-ketosteroids, particularly a step beyond the first change in the testosterone molecule.

The limiting reaction in the cirrhotic patients did not appear to be the conjugation reaction. If the decreased 17-ketosteroid excretion in patients with liver disease were caused by a decrease in the rate of conjugation, it would be expected that free 17-ketosteroids

would accumulate in the tissues and blood, assuming that the 17-ketosteroids were not subjected to further metabolic breakdown. No evidence of increased free 17-ketosteroids in the urine or plasma of cirrhotic patients was found. It is possible that the 17-ketosteroids accumulated in the tissues and were not detected but this seems highly unlikely since then a higher than normal excretion would have been expected as the 17-ketosteroids returned to the circulation.

This decrease in the usually preferred path of testosterone metabolism apparently results in an increased disappearance by other routes, for much loss of the injected hormone could be accounted for by increased excretion of 17-ketosteroids. The total excess 17-ketosteroid excretion in patients with liver disease is only approximately 1/3 of that in normals, with a large degree of variation depending upon the severity of the liver disease. This indicates that in liver disease a large part of the administered testosterone is metabolized to other metabolites than 17-ketosteroids. These may be steroidal or non-steroidal. Unfortunately this investigation made no attempt to establish the urinary steroid pattern in the patients with cirrhosis by identification of the excreted steroids, nor were the feces analyzed.

The kidney is known to contain enzymes capable of metabolizing testosterone. Yet in kidney disease, 17-ketosteroid formation still occurred at a rapid rate after the intravenous injection of testosterone, as evidenced by the very high 17-ketosteroid levels in plasma. Obviously the exact role of the kidney in relation to the liver in the over-all metabolism of testosterone by the whole body cannot be determined from these data. The experiments with hepatectomized, nephrectomized

animals injected intravenously with testosterone would indicate that other tissues besides liver and kidney must be capable of metabolizing testosterone. No way of testing this hypothesis in human subjects was found in these experiments.

SUMMARY

The fate of testosterone administered intravenously in experimental animals has been investigated. The injected testosterone diffused rapidly from the circulation into various tissues of the body, the highest concentrations being found in fat. The testosterone was apparently not metabolized in the fatty tissues but was reabsorbed back into the circulation as it was removed by other structures. Evidence is presented to indicate that the liver and kidney are probable sites of such metabolism. That other sites than liver and kidney might be involved in the metabolism of testosterone was indicated by studies in hepatectomized, nephrectomized animals in which less than 50% of the testosterone injected could be accounted for in a major proportion of the tissues.

The metabolism of testosterone in human serum albumin given intravenously to human subjects was studied. In normal subjects an average of 74% of the injected testosterone was accounted for by an increase in urinary conjugated 17-ketosteroids during the post-injection period. An average of 63% was excreted during the first 24 hours with the major part of this excretion occurring during the first two hours. Following intravenous testosterone conjugated 17-ketosteroids were demonstrable in the plasma. Maximum plasma levels were found at 20 minutes after administration.

The importance of the liver in the metabolism of testosterone was demonstrated by the lower concentrations of 17-ketosteroids in both the urine and plasma following administration of testosterone intravenously

in a series of patients with liver disease. More than one pathway of testosterone metabolism was indicated by the much lower proportion of the injected hormone which was recovered as excess 17-ketosteroids in the cirrhotic patients in spite of the rapid disappearance and minimal excretion of the original steroid.

After intravenous administration of testosterone to normal males the following steroids were isolated from the urine and identified: etiocholanol-3α-one-17, androsterone, etiocholanediol 3α,17α and $\Delta^{3,5}$androstadienone-17. It was further demonstrated that the androsterone and etiocholanolone which made up the greater part of the 17-ketosteroids in the urine were conjugated with glucuronic acid. No significant amounts of 3β-hydroxysteroids were found in the ketonic fraction.

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